Universidade de Lisboa
Faculdade de Medicina de Lisboa

Effects of chemotherapy and radiotherapy in rectal cancer: significance of different cellular outcomes in tumor behavior

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Doutoramento em Ciências Biomédicas

Especialidade de Ciências Biopatológicas

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“Pensar é mais interessante que saber, mas menos interessante que olhar.”
Johann Goethe

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UNITS OF MEASUREMENT AND ABBREVIATIONS

(In alphabetic order)

%	Percent
°C	Degree Celsius
µg	Microgram
µM	Micro molar
µm	Micrometer
53BP1	p53-Binding Protein 1
5-FU	5-fluorouracil
9-1-1	RAD9–RAD1–HUS1 Protein Complex
Ang	Angiogenin
AREG	Amphiregulin
Arf	Alternative Reading Frame
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related
BRCA1	Breast Cancer 1
BrdU	Bromodeoxyuridine
CDC25	Cell Division Cycle 25
Cdk	Cyclin Dependent Kinase
Cells/ml	Cells per Milliliter
CH_2THF	5,10-Methylenetetrahydrofolate
CHK2	Checkpoint Kinase 2
CM	Conditioned Medium
CO_2	Carbon dioxide
Cpss	Steady-State Serum Concentrations
CRC	Colorectal cancer

CRT	Chemoradiotherapy
CT	Chemotherapy
CT+ Bev	Chemotherapy plus Bevacizumab
CT+PBO	Chemotherapy plus Placebo
DAPI	Fluorescent Stain - 4',6-diamidino-2-phenylindole
DCR2	Decoy Receptor 2
DDR	DNA Damage Response
DEC1	Basic Helix–loop–Helix Transcription Factor
DFS	Disease Free Survival
DHFU	Dihydrofluorouracil
Dkk-1	Dickkopf-related protein 1
DNA	Deoxyribonucleic Acid
DNase I	Deoxyribonuclease I
DPD	Dihydropyrimidine Dehydrogenase
DSBs	Double Strand Breaks
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
EORTC	European Organization for Research and Treatment of Cancer
ESMO	European Society for Medical Oncology

FdUMP	Fluorodeoxyuridine Monophosphate
FdUTP	Fluorodeoxyuridine Triphosphate
FFPE	Formalin-Fixed Paraffin-Embedded
FGF-19	Fibroblast Growth Factor 19
FGF7	Fibroblast Growth Factor 7
FUdR	5-Fluoro-2'-Deoxyuridine
FUTP	Fluorouridine Triphosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GROα	Growth Regulated Oncogene-alpha
H3K9me3	Trimethylation of Lysine 9 in Histone 3
HCT 116	Homo sapiens Colorectal Carcinoma Cell Line
HGF	Hepatocyte Growth Factor
HIRA	Histone Repressor A
HP1γ	Heterochromatin Protein 1-γ
HPF	Hepatocyte Growth Factor
IARC	International Agency for Research on Cancer
IC50	Half Maximal Inhibitory Concentration
IF	Immunofluorescence
IGFBP	Insulin-like Growth Factor Binding Protein
IGFBP2	Insulin-like Growth Factor-Binding Protein 2
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
Iv	Intravenous

KO	Knockout
LCN2	Lipocalin-2
M	Molar
MDC1	Mediator of DNA Damage Checkpoint 1
MDM2	E3 Ubiquitin-Protein Ligase
MEFs	Mouse Embryonic Fibroblasts
$Mg/m^2/day$	Milligram per Square Meter per Day
$MgCl^2$	Magnesium chloride
MIC 1	Macrophage Inhibitory Cytokine 1
MIF	Macrophage Migration Inhibitory Factor
ml	Milliliter
mM	Milimolar
mm	Millimeter
MMPs	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MRN	MRE11–RAD50–NBS1 Protein Complex
mRNA	Messenger Ribonucleic Acid
Neo.CRT	Neoadjuvant Chemoradiotherapy
nm	Nanometer
Non-SAS -medium	Conditioned medium from non-senescent cells
NSABP R-03	National Surgical Adjuvant Breast and Bowel Project R-03
OS	Overall Survival
PAI-1	Plasminogen Activator Inhibitor-1
PBS buffer	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05 % Triton x-100

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF-AA	Platelet-Derived Growth Factor AA
PFA	Paraformaldehyde
PFS	Progression Free Survival
pg/mL	Pico gram per Milliliter
pH	Power of Hydrogen
PML	Promyelocytic Leukemia
pRb	Retinoblastoma Protein
RNA	Ribonucleic Acid
RNase A	Ribonuclease A
RPA	Replication Protein A
RT	Radiotherapy
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
RT-qPCR	semi-quantitative real-time PCR
SAHF	Senescence-Associated Heterochromatic Foci
SAS	Senescence-Associated Secretome
SAS-medium	Conditioned medium from senescent cells
SASP	Senescence-Associated Secretory Phenotype
SA-β-Gal	Senescence-Associated β- Galactosidase
SEM	Standard Error Mean
SSB	Single Strand Break
TGF-α	Transforming Growth Factor alpha
TIS	Therapy-Induced Senescence

TMP	Thymidine Monophosphate
TOPBP1	Topoisomerase-II-Binding Protein 1
TP	Thymidine Phosphorylase
TRG	Tumor Regressing Grade
TS	Thymidylate Synthase
TTP	Time to Progression
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
U/ml	Units per milliliter
uPAR	Urokinase-Plasminogen Activator Receptor
Uv	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl β-d-Galactopyranoside
γH2AX	Phosphorylation of the histone variant H2AX on Ser139
µg/ml	Microgram per Milliliter

SUMMARY

Rectal cancers comprise 35% of all diagnosed colorectal cancers (CRC), being the third most common among gastrointestinal cancers. Despite the benefits of neoadjuvant chemoradiotherapy (CRT), 5-fluorouracil (5-FU)-based regimens plus radiotherapy (RT), 15-20% of patients suffer from relapse. Pathological staging remains the most important prognostic factor in rectal cancer and the search for new prognostic and predictive biomarkers is fundamental. DNA damaging agents and ionizing radiation used in the therapy of human cancers may induce senescence of cancer cells. Senescent cells exhibit a secretory phenotype that can affect cancer cell behavior and, eventually, clinical prognosis. **In this work we hypothesize that neoadjuvant CRT-induced cellular senescence may affect rectal cancer relapse**. To experimentally test our hypothesis, we cultured colon cancer cells induced into senescence by exposure to 5-FU or doxorubicin. SAS-media were enriched in IL-8, TGF-α, VEGF, cystatin C, LCN2, MIF, EMMPRIN, and uPAR, and exerts a positive effect on the proliferation of cycling colon and rectal cancer cells. SAS-medium was capable of paracrine induction of epithelial-to-mesenchymal (EMT) transition in colon and rectal cancer cell lines, of increased cell invasion *in vitro*, and of increased chemosensitivity to 5-FU. Moreover, we found that in rectal cancer samples from patients treated with neoadjuvant CRT tumor cell niches enriched for senescent cells bookmark regions of increased expression of EMT-related genes (*slug*, *snail*, *vimentin*) when compared to nearby senescent-null control regions. We provide evidences that therapy-induced senescent cancer cells influence the tumor microenvironment by promoting EMT via short range interactions. Next, to relate neoadjuvant CRT-induced cellular senescence with rectal cancer relapse, we retrospectively studied rectal cancers from 35 patients treated with neoadjuvant therapy. Data showed no correlation between the senescence markers $p16^{INK4a}$ and $p21^{WAF1}$ and relapse, and it was not possible to validate a method for senescence detection in formalin-fixed and paraffin-embedded (FFPE) samples. Altogether, our findings showed that secretomes from senescent colon cancer cells may induce effects with opposite prognostic value. Prospective studies shall clarify whether, after neoadjuvant therapy, the presence of senescent cells add prognostic power on cancer recurrence and patient survival.

Keywords: Therapy-induced cellular senescence; rectal cancer; neoadjuvant chemotherapy; senescence-associated secretory phenotype; 5-fluorouracil

RESUMO

O cancro do reto representa cerca de 35% de todos os tumores colo-retais (CCR) diagnosticados, sendo o terceiro mais comum entre os tumores gastrointestinais. Apesar da melhoria significativa no contexto da terapia neoadjuvante, 5-fluorouracil (5-FU) em concomitância com radioterapia (RT), as taxas de recidiva permanecem elevadas, cerca de 15-20%. O estadiamento histo-patológico permanece o principal fator de prognóstico sendo fundamental a pesquisa de novos biomarcadores de prognóstico. No tratamento da doença oncológica, a terapêutica com citostáticos e radiação pode induzir senescência cujo fenótipo secretor é conhecido por *senescence-associated secretory phenotype* (SASP), capaz de modificar o micro-ambiente e contribuir para a progressão tumoral. **Este trabalho teve por base a hipótese de que a senescência celular induzida pela quimioterapia (QT) pode estar correlacionada com a recidiva em doentes com cancro do reto em estadio avançado.** Para testar experimentalmente esta hipótese foi induzida senescência em células de cancro do cólon (HCT 116) por exposição ao 5-FU ou doxorrubicina. A análise dos meios condicionados obtidos (SAS-*media*) revelou a presença dos compostos IL-8, MIF, VEGF, uPAR, EMMPRIN, cistatina C, lipocalina-2 e TGF-α, que se verificou estimularem a proliferação de células de cancro do cólon e reto não senescentes. *SAS-medium* induziu também um aumento significativo da expressão de marcadores moleculares de transição epitélio-mesênquima (TEM), do potencial invasivo em células tumorais não senescentes, e da quimio-sensibilidade, o que sugere um duplo efeito nas células tumorais não senescentes. Em amostras humanas de tumores do reto verificamos um aumento da expressão de marcadores mesenquimais em zonas tumorais associadas à presença de células senescentes, constituindo uma forte evidência de que a senescência induzida pela QT influencia de facto o micro-ambiente tumoral, promovendo a TEM. Para determinar uma possível correlação entre a presença de células senescentes e a recidiva no cancro do reto, realizámos um estudo retrospetivo envolvendo 35 amostras de doentes submetidos a terapia neoadjuvante. Os dados obtidos não permitiram estabelecer uma correlação entre os marcadores de senescência $p16^{INK4a}$ e $p21^{WAF1}$ e a recidiva, não tendo sido possível validar um método que consideremos adequado para a identificação de células senescentes em amostras fixadas. Em conclusão, os dados obtidos neste trabalho sugerem que o secretoma de células epiteliais tumorais pode induzir fenómenos com valor prognóstico opostos. Estudos prospetivos futuros irão contribuir para clarificar o papel da senescência celular na definição de novos critérios de prognóstico e identificação de novos alvos terapêuticos.

Palavras-chave: Senescência celular induzida pela terapia; cancro do reto; quimioterapia neoadjuvante; secretoma associado à senescência; 5-fluorouracil.

SCOPE OF THE THESIS

This thesis is organized in four chapters. In **Chapter 1** the background, significance, and aims of this study are presented. Rectal cancer epidemiology and clinical management; the mechanism of action of 5- fluorouracil; and the molecular basis and significance of cellular senescence, were reviewed according to the literature. Together, this review comprises the theoretical fundaments that support our hypothesis that therapy-induced cellular senescence can be related with rectal cancer relapse. In **Chapters 2 and 3** are presented and discussed the results obtained during the course of this work **(Illustration 1)**. **Chapter 2** refers to a manuscript submitted to *Clinical & Experimental Metastasis* (CLIN-S-14-00188, submitted on September 11, 2014), entitled "*Therapy-induced cellular senescence has a dual effect in rectal cancer increasing invasiveness and chemosensitivity",* by Joana Tato-Costa, Sandra Casimiro, Teresa Pacheco, Ricardo Pires, Afonso Fernandes, Irina Alho, Pedro Pereira, Paulo Costa, Henrique Bicha Castelo, João Ferreira, and Luís Costa. **Chapter 3** describes a clinicopathologic study conducted to elucidate if cellular senescence could be correlated with relapse in rectal cancer. Finally, in **Chapter 4** it is presented a final discussion of the key findings from the previous chapters and of the potential translational relevance of senescence in the management of advanced rectal cancer.

Disclaimer: I hereby declare that I majorly contributed for the conception and design, development of methodology, acquisition of data, analysis and interpretation of data and writing, review and/or revision of each group of results and chapters of this thesis.

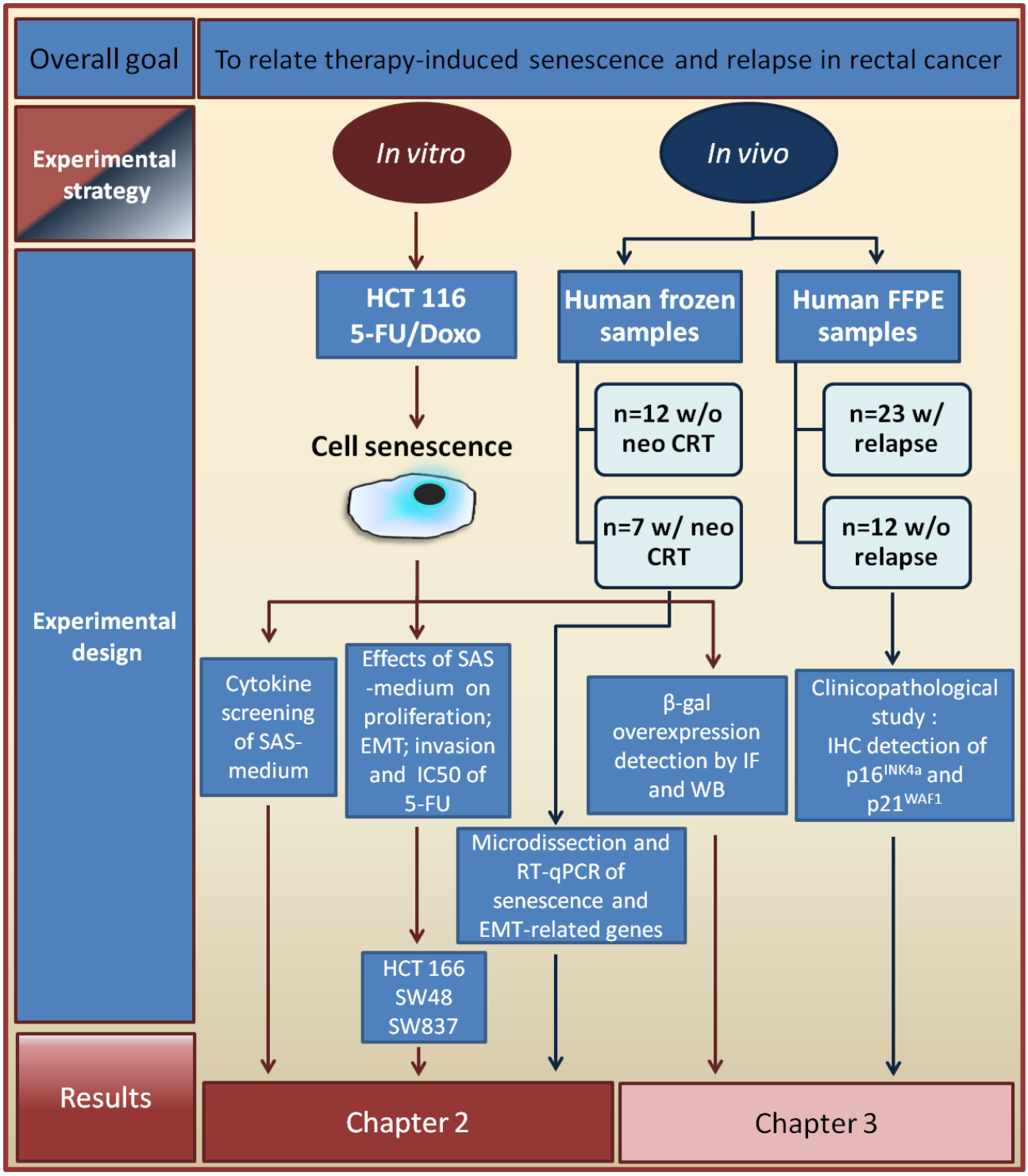

Illustration 1- Conceptual framework used for the investigation of the effects of cellular senescence in rectal cancer.

HCT 116; SW48 – colon cancer cell lines and SW837 - rectal cancer cell line, all acquired from ATCC; SAS-medium – culture medium enriched of the Senescence-Associated Secretome; EMT - Epithelial-Mesenchymal Transition; W/O – Without; W/ - With; CRT –Chemoradiotherapy; RT-qPCR - Semi-quantitative real-time PCR; β-gal - β-galactosidase; IF – Immunofluorescence; WB – Western blot; FFPE - Formalin-Fixed, Paraffin-Embedded; IHC – Immunohistochemistry.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Incidence and classification of colorectal cancer

Colorectal cancer (CRC) is the third most common form of cancer worldwide. In 2012, according to the GLOBOCAN Project, sponsored by the International Agency for Research on Cancer (IARC) and World Health Organization (WHO), CRC represented 9.7% of the world's new cancers with 1.36 million new cases diagnosed [5].

In Portugal, in the same period, 7 129 new cases were diagnosed with CRC, corresponding to 14.5% of all diagnosed cancers. Here, CRC was the second most common cancer affecting women (after breast cancer) and men (after prostate cancer), and had the highest mortality rate (15.7%) [5].

Staging of the disease dictates the therapeutic strategies. According to international guidelines from the European Society for Medical Oncology (ESMO), the preoperative staging of CRC is based on the clinical and radiological evaluation of the tumor growth and distant spread [6, 7]. Following surgery, the pathologic TNM classification is based on the evaluation of size and extent of the primary tumor (ranging from T0 to T4), the presence of regional lymph nodes involvement (N0 to N2), and the existence of distant metastasis (M0 or M1) **(Table 1.1)** [8].

CRC can occur at different locations within the colon or rectum. Rectal cancer is the third most common among gastrointestinal (GI) cancers, and represents approximately 35% of all CRC cancers [6]. Rectal cancers are located below 12 cm from the anal verge as measured with a rigid rectoscope, or below the pelvic promontory as visualized by X-ray, computed tomography, magnetic resonance imaging (MRI) or during surgery [7]. Colon and rectal cancers have several features in common and are epidemiologically gathered as CRC. However, the surgical and therapeutic management of colon and rectal cancers is different. This thesis and following chapters will focus on rectal cancer.

Table 1.1- TNM Classification system for colorectal cancers [8].

TNM system		
T- Primary tumor	**Tx**- Primary tumor cannot be assessed	
	T0- No evidence of primary tumor	
	Tis- Carcinoma *in situ*: intraepithelial or invasion of lamina propria	
	T1- Tumor invades submucosa	
	T2- Tumor invades muscularis propria	
	T3- Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissue	
	T4- Tumor directly invades other organs or structures and/or perforates visceral peritoneum	**T4a**- Tumor perforates visceral peritoneum **T4b**- Tumor directly invades other organs or structures
N- Regional lymph nodes	**Nx**- Regional lymph nodes cannot be assessed	
	N0- No regional lymph nodes metastasis	
	N1- Metastasis in 1-3 regional lymph nodes	**N1a**- Metastasis in 1 regional lymph node **N1b**- Metastasis in 2-3 regional lymph nodes **N1c**- Tumor deposit(s), i.e., macro or microscopic nests or nodules in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue without regional lymph nodes metastasis
	N2- Metastasis in 4 or more regional lymph nodes	**N2a**- Metastasis in 4-6 regional lymph nodes **N2b**- Metastasis in 7 or more regional lymph nodes
M- Distant metastasis	**M0**- No distant metastasis	
	M1- Distant metastasis	**M1a**- Metastasis confined to 1 organ **M1b**- Metastasis in more than 1 organ or the peritoneum

1.2 Rectal Cancer

1.2.1 Treatment of locally advanced primary rectal cancer

The standard of care treatment for locally advanced rectal cancer (stage T3/T4 and/or N positive) comprises preoperative (neoadjuvant) chemoradiotherapy (CRT), followed by surgery and postoperative (adjuvant) chemotherapy (CT) **(Figure 1.1)**.

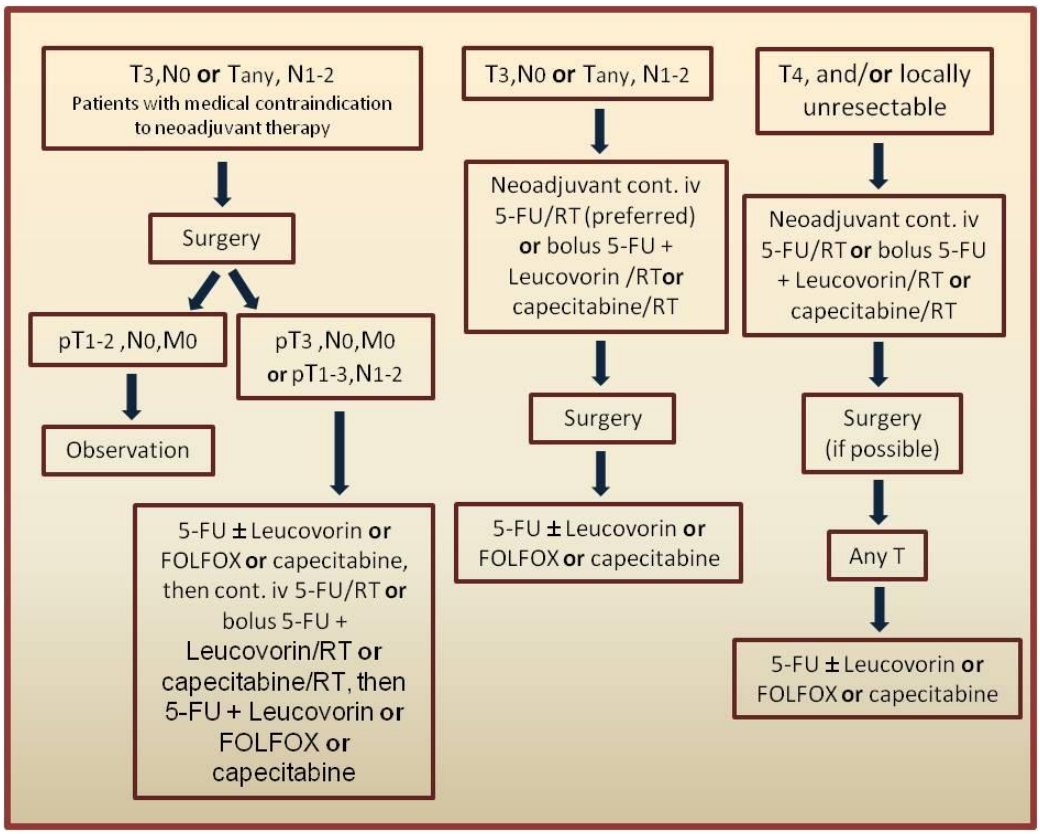

Figure 1.1- Representative scheme of current guidelines for the treatment of locally advanced rectal cancer.

5-FU -5-fluorouracil; Leucovorin- Folinic acid; FOLFOX- Chemotherapy regimen comprising folinic acid (Leucovorin), 5- fluorouracil (5-FU) and oxaliplatin; RT- radiotherapy; cont. iv- continuous intravenous infusion. Adapted from [9].

New surgical techniques have substantially improved local-regional control and overall survival[1] (OS), being total mesorectal excision the gold standard procedure, with local failure rates below 10% and better prevention of tumor cells spread at surgery [10].

[1] Overall survival (OS) - The length of time those patients are alive since either the date of diagnosis or the start of treatment for a disease.

However, and despite surgical and therapeutic advances, 15 to 20% of patients with locally advanced rectal cancer will suffer from local or distant relapse, mainly to the liver, lung and distant lymph nodes. In consequence, about one third of the diagnosed patients will die within 5 years after treatment [11].

In 2006, results from The European Organization for Research and Treatment of Cancer (EORTC) 22921 multicentre clinical trial, enrolling 1011 patients with primary T3/T4 rectal cancers, suggested that a pre-surgical treatment with radiotherapy (RT) combined with radiosensitive CT could significantly improve downsizing and downstaging of tumors and decrease local recurrence rates, when compared to RT alone (57.1% *versus* 42.4%) [11].

Results from the National Surgical Adjuvant Breast and Bowel Project R-03 (NSABP R-03) trial, comparing the effects of neo plus adjuvant CRT in patients with locally advanced rectal adenocarcinoma, showed that the five year disease free survival[2] (DFS) was significantly improved for patients treated with neo plus adjuvant therapy (65% *versus* 53%, $p = 0.011$). In addition, the five year OS for patients treated with neo plus adjuvant therapy was significantly higher in comparison with patients with only post-operative therapy (75% *versus* 66%, $p = 0.065$) [12].

Different studies have demonstrated that neoadjuvant CRT not only improves surgical outcomes, but also reduces tumor burden and RT-related toxicity, and increases radiosensitivity [13, 14].

The advent of targeted therapy with target-specific monoclonal antibodies, namely cetuximab and bevacizumab, in combination with CT expanded the treatment options and further improved clinical outcomes. However, these improvements are so far only reported in cases of metastatic CRC [15].

Cetuximab is a monoclonal antibody that binds to Epidermal Growth Factor Receptor (EGFR) and blocks ligand-induced phosphorylation of the downstream effectors of the pathway. The overexpression or up-regulation of EGFR is associated with poor survival in

[2] Disease-free survival (DFS) - the period of time after primary treatment in which the patient does not show any signs or symptoms of the disease.

CRC and occurs in 60 to 80 % of CRCs. Upon activation, EGFR activates an important signaling pathway that regulates cell differentiation, proliferation, migration, angiogenesis, and apoptosis, all of which become deregulated upon over expression of the receptor. A clinical trial where was compared the combination of cetuximab and irinotecan (the semi-synthetic topoisomerase I inhibitor camptothecin used as CT drug) with cetuximab in monotherapy in metastatic CRC cancer patients showed that the response rate in the combination-therapy group was significantly higher than that in the monotherapy group (22.9% *vs*. 10.8%, p=0.007). The median time to progression[3] (TTP) was significantly improved in the first group (4.1 *vs.* 1.5 months, P<0.001) although without significant differences in median OS (8.6 versus 6.9 months, p=0.48). Cetuximab has proved to be clinically significant when given in combination with irinotecan in patients with metastatic disease [16].

Bevacizumab is a monoclonal antibody against Vascular Endothelial Growth Factor (VEGF), an important regulator of physiologic and pathologic angiogenesis. Bevacizumab has antiangiogenic effects and currently being evaluated in clinical trials as a treatment for several cancers. In a phase 3 clinical trial the addition of bevacizumab to a standard combination of three CT drugs (CT+bev) improved OS among patients with metastatic CRC when compared to CT plus placebo (CT+PBO) (20.3 versus 15.6 months, p<0.001). The median progression free survival[4] (PFS) was 10.6 months *vs.* 6.2 months (p<0.001) and the corresponding rates of response were 44.8% *vs.* and 34.8% (p=0.004) [17].

1.2.2 Prognostic and predictive factors

The major prognostic factors in rectal cancer are: clinical staging before surgery and pathologic tumor staging after surgery, including: the presence of residual tumor; the number of positive lymph nodes; and tumor differentiation grade. In addition, vascular and nodal invasion; the number of positive lymph nodes; perineural growth; and invasion of adjacent organs, are also important prognostic factors [18-20].

[3] Time to progression (TTP) - the period of time from the date of diagnosis or the start of treatment for a disease until the progression of the disease.

[4] Progression-free survival (PFS) - the period of time between treatment initiation and tumor progression or death from any cause.

The pathologic response assessed by the tumor regressing grade (TRG) after pre-operative CRT, has also been considered a valuable tool to predict prognosis and long-term survival [21]. This system is based on the determination of the amount of viable tumor cells versus fibrosis, ranging from TRG4 (when no viable tumor cells are detected) to TRG0 (when fibrosis is completely absent). Despite some evidences that indicate TRG as an independent prognostic factor and predictor for long-term outcome after preoperative treatment [22], only pathological staging (TNM) has been validated in multi-institutional prospective studies and remains the main prognostic tool for rectal cancer.

Mutational profiling of specific genes has also been reported as useful in predicting treatment response and survival in patients with metastatic CRC. Mutation(s) in K-*ras*, *PIK3CA,* and *BRAF* showed to be highly associated with treatment response to targeted therapy with monoclonal antibodies anti-EGFR as well as to the clinical outcome of the patients [23]. The most clinically relevant example relates with K-*ras* mutations and poor responses to EGFR monoclonal antibodies like cetuximab. In fact, patients with mutations in K-*ras* are unlikely to benefit from anti-EGFR therapy and are significantly associated with resistance to cetuximab ($p< 0.001$) and a lower OS (10.1 *versus* 14.3 months in patients without mutation*;* $p = 0.026$) [24].

1.3 5-fluorouracil (5-FU)

1.3.1 Mechanism of action

5-fluorouracil (5-FU) has been used for more than 40 years to treat cancer and is still recommended as a first-line anticancer drug for patients with locally advanced rectal cancer in both neo and adjuvant treatment setting [25]. Since 5-FU can act as a radio sensitizer at the cellular level, it has been extensively used concomitantly with radiation [26].

5-FU is a fluoropyrimidine antimetabolite most frequently administrated by continuous intravenous (Iv) infusion (225–300 mg/m^2/day), or alternatively in its oral form – Capecitabine [13].

5-FU rapidly enters the cells where is converted into three active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP), which have different effects. FdUMP inhibits the action of thymidylate synthase (TS), FUTP disrupts RNA synthesis, and FdUTP is directly misincorporated into DNA **(Figure 1.2)** [27].

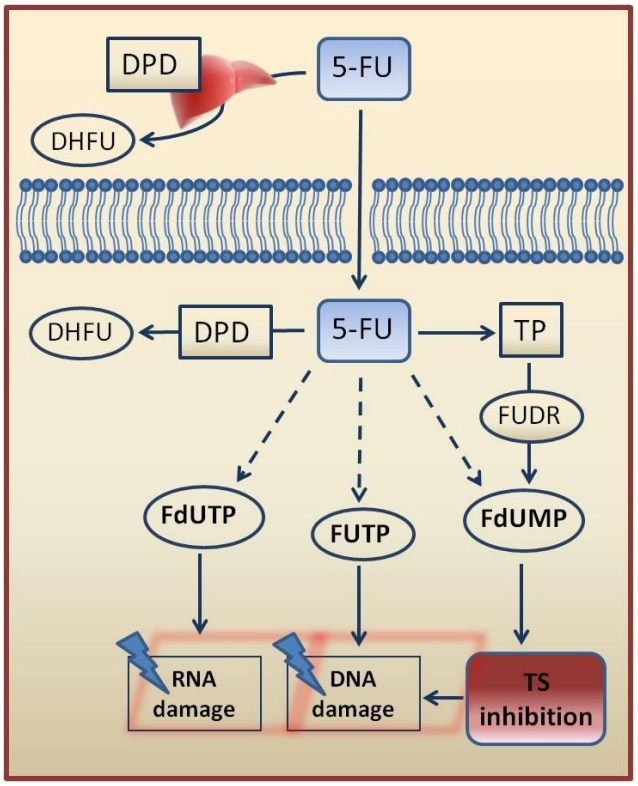

Figure 1.2- Representative scheme of 5-Fluorouracil metabolism.

5-FU is taken up into cells and converted into three main active metabolites: fluorodeoxyuridine triphosphate (FdUTP), fluorouridine triphosphate (FUTP) and fluorodeoxyuridine monophosphate (FdUMP). An alternative activation pathway involves the thymidine phosphorylase (TP) catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated and converted into FdUMP. 5-FU can inhibit RNA synthesis in a pathway that involves its transformation into FdUTP, can cause DNA damage by directly misincorporation of FUTP and by FdUMP binding to the nucleotide-binding site of thymidylate synthase (TS), inhibiting DNA synthesis. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells. Up to 80% of administered 5-FU is metabolized by DPD in the liver. Adapted from [3].

TS is a nucleotide synthetic enzyme, that catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with the reduced folate 5,10-methylenetetrahydrofolate (CH_2THF) as the methyl donor, creating the only *de novo* source of thymidine monophosphate (TMP), which is necessary

for DNA replication and repair [28, 29]. Due to its crucial importance to DNA replication, TS is an important target for fluoropyrimidine drugs, such as 5-FU.

TS contains a nucleotide-binding site and a binding site for CH_2THF. The 5-FU metabolite FdUMP binds covalently to the active site of TS forming a stable ternary complex with the enzyme and with CH_2THF, blocking the binding of the normal substrate dUMP thereby inhibiting dTMP synthesis. As a consequence, there is a depletion of dTMP which induces deoxynucleotides disequilibrium, restricting proper DNA replication and repair mechanisms, resulting in DNA damage and inducing cell-cycle arrest **(Figure 1.3)** [3, 30].

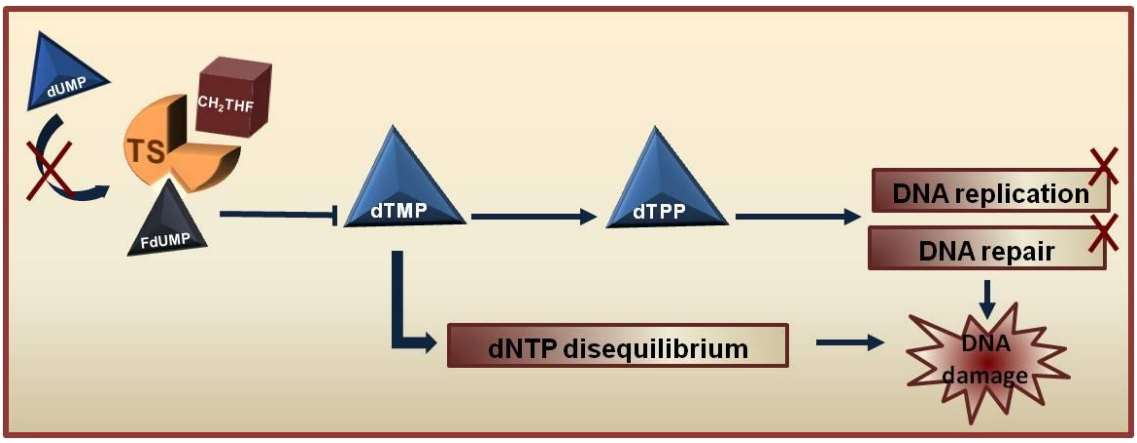

Figure 1.3- Mechanism of thymidylate synthase (TS) inhibition by 5-Fluorouracil.

Thymidylate synthase (TS) catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH2THF) as the methyl donor. In the presence of the 5-FU active metabolite FdUMP it binds to the nucleotide-binding site of TS and forms a stable ternary complex with TS and CH2THF, blocking access of dUMP and inhibiting dTMP synthesis. This results in deoxynucleotide (dNTP) disequilibrium and DNA damage. Adapted from [3].

Other relevant enzymes involved in 5-FU metabolism are dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) **(Figure 1.2)**. DPD is the rate-limiting enzyme in 5-FU catabolism which converts 5-FU to dihydrofluorouracil (DHFU). Over 80% of the administered 5-FU is normally catabolised primarily in the liver, where DPD is abundantly expressed [31]. TP function is related with an indirect metabolization pathway of 5-FU to FdUMP by 5-fluoro-2'-deoxyuridine (FUdR).

1.3.2 Chemo-resistance to 5-fluorouracil

Poor response rates to CT are mainly due to the acquisition of resistance mechanisms. Chemo-resistance is believed to be the main cause of treatment failure in around 90% of patients with metastatic malignant diseases [32]. Therefore, it is important to characterize the biological factors that are involved in these mechanisms since they are directly correlated with treatment responses.

Regarding 5-FU, the expression of TS influences its therapeutic efficacy. In gastric cancer and CRC, data showed that patients whose tumors did not respond to treatment had the mean TS protein level significantly higher (14.5 *versus* 1.36, $p< 0.01$) comparing with samples from patients with responsive disease. The same result was achieved concerning the expression levels of TS messenger RNA (mRNA) (0.17 *versus* 0.60 arbitrary units, $p< 0.01$). Thus high expression and protein levels were correlated with lack of response to 5-FU [33]. In accordance, other studies show that low tumoral TS expression improves the efficacy of 5-FU based therapies, since TS expression was significantly associated with OS ($p= 0.002$) [34, 35].

TS protein expression is regulated at the post-transcriptional level, by inhibition of a negative feedback mechanism in which ligand-free TS binds to its own mRNA inhibiting its own translation. When stably bound to FdUMP, TS can no longer bind to the mRNA, maintaining a constant level of free enzyme [27, 36]. This suggests that the amount of FdUMP and the acute increase in TS expression (that facilitates the recovery of the enzyme activity) in response to cytotoxic agents, may play a role in the development of an important resistance mechanism [33].

Also in CRC, low levels of DPD mRNA expression were found in all 5-FU responders compared with higher levels in the 5-FU non-responders. Furthermore, few studies also demonstrated that high levels of TP mRNA also correlates with resistance to 5-FU [37]. However, there is no correlation between TS, DPD, and TP expression values in CRC, indicating that they are independent predictive markers of 5-FU response [38].

1.3.3 5-FluorouracIl pharmacokinetics

Regarding 5-FU pharmacokinetics, parameters like steady-state concentrations (Cpss) and total body clearance have been studied in order to determine the benefits in terms of clinical response and toxicity obtained by prolonging the drug delivery period and/or increasing the blood concentration [39].

Following a continuous drug infusion, the concentration of the drug in the plasma will increase until the rates of drug administration and drug elimination are equal. When the plasma concentration is constant Cpss is reached.

Cpss of 5-FU in plasma, measured in patients after continuous 5-FU infusion of 200 mg/m^2/day ranged from 0,39 µM to 66,3 µM [40, 41]. This fact is extremely important, since it has been reported that standard CT regimens, are not only cytotoxic but also able to induce cellular senescence, a state of irreversible proliferative arrest [42, 43]. This means that at low levels CT drugs can have different effects on the tumor cells.

1.4 Cellular senescence

Senescence derives from the Latin word *senescere* that means 'to grow old'. When applied to life sciences, senescence was first defined as a set of deteriorative processes resulting from biological aging, where an organism progressively accumulates changes in their molecular and cellular structure, consequently altering their metabolism and resulting in death [44, 45]. However, the biological concept of senescence has evolved substantially.

In 1961 Hayflick and Moorhead [46] described for the first time that normal somatic cells have limited proliferation ability, and that cells that initially proliferate at a normal rate gradually lose the ability to proliferate despite optimal culturing conditions. These results were later confirmed, leading to the hypothesis that cellular senescence is the cell mechanism responsible for the finite life time of human diploid cells [47].

The process observed by Hayflick is named replicative or cellular senescence, and occurs due to the progressive shortening of the telomeres in proliferating cells, leading to a proliferation limit. The first report suggesting that telomere shortening is the major mechanism responsible for replicative senescence came in early 1990s [48] and it is

currently the only known endogenous mechanism of senescence induction. The majority of normal cells, from all vertebrate species, undergo replicative senescence, although the number of cell divisions before senescence can be relatively variable [49].

Telomeres are regions of tandemly repeated hexanucleotide sequences (5'-TTAGGG-3' in vertebrates). Many cellular factors directly (e.g. TRF1/TRF2) and indirectly (e.g. shelterin-complex, PinX, Apollo and tankyrase) interact with telomeres, influencing telomere structure and function [50]. Located at the ends of chromosomes, these regions prevent cells from recognizing chromosome ends as double strand breaks (DSBs), avoiding the fusion with other chromosomes ends by DNA-repair mechanisms [51]. Therefore, telomeres are essential to maintain the stability and integrity of eukaryotic genomes [52]. During replication, DNA polymerase fails to completely replicate DNA ends, a process known as the "end-replication problem", which contributes to telomere shortening. As a result, cells lose between 50 to 200 base pairs of telomeric DNA during each round of DNA replication [51]. When telomeres reach a critically short length, their protective function is disrupted, triggering a sustained DNA damage signal that will eventually cause the cell to stop dividing.

Cancer and germline cells, unlike most somatic adult cells, express telomerase, a cellular reverse transcriptase whose function is to add telomeric DNA to the chromosome ends, elongating the telomeres and solving the problem of the "end-replication" [52]. Telomerase however, either endogenous or ectopically expressed (to immortalize primary cells *in vitro*), does not completely impair senescence since it can be induced by multiple extrinsic factors besides telomere erosion. This type of telomere-independent senescence has been termed stress-induced or premature senescence, as it is established prior to telomere shortening-induced senescence [53].

Currently, the concept of cellular senescence is applied to any type of irreversible proliferation arrest. Multiple extrinsic factors such as DNA damaging agents (e.g. ionizing radiation and chemotherapy agents), oxidizing agents, and oncogene activation, have been found to trigger telomere-independent senescence [54-57]. Therefore, 'replicative senescence' refers to telomere-derived senescence and 'stress-induced (or premature) senescence' (SIS) refers to senescence induced by exogenous factors [2].

1.4.1 Signaling pathways mediating senescence

Independently of the trigger factor, senescence state derives from DNA damage response (DDR) activated signalling cascade, due to the occurrence of single strand breaks (SSB) or double strand breaks (DSB).

DDR is crucial to preserve and maintain genome integrity. Besides activation of DNA repair pathways, DDR can also induce the arrest of cell cycle progression by activating checkpoint proteins. This allows the cell time to repair the DNA damage, preventing the replication of damaged DNA.

The DDR **(Figure 1.4)** pathway is activated by two major sensors of damage: the MRE11–RAD50–NBS1 (MRN) complex, which detects DNA DSBs, and replication protein A (RPA) that, together with RAD9–RAD1–HUS1 (9-1-1) complex, which detects exposed regions of single-stranded DNA. These damage sensors recruit apical kinases according to the type of lesion. DSBs signal leads to the activation of ataxia-telangiectasia mutated (ATM), whereas single-stranded DNA regions engage ataxia telangiectasia and Rad3-related (ATR) [58]. The recruitment of ATM or ATR leads to phosphorylation of histone variant H2AX on Ser139 (known as γH2AX). In the case of DSBs, γH2AX is required to activate mediator of DNA damage checkpoint 1 (MDC1) and p53-binding protein 1 (53BP1) that amplify the DDR by recruiting additional ATM complexes in a positive feedback loop. In the case of single-stranded DNA damage, ATR kinase activity is enhanced by the 9-1-1 complex and by topoisomerase-II-binding protein 1 (TOPBP1). Breast cancer 1 (BRCA1) is then phosphorylated by ATM or ATR, and recruited to the sites of DNA damage [4, 59]. After amplification of the DDR signal, ATM and ATR activate by phosphorylation the checkpoint kinases 2 (CHK2) and CHK1, respectively. CHK2 and CHK1 will establish the communication between DDR-associated factors and the cell cycle machinery through phosphorylation and activation of downstream effectors such as p53 and the cell division cycle 25 (CDC25) phosphatases [4, 53]. The interaction between the ATM and ATR signalling pathways is complex. The ATR-Chk1 pathway can be activated in response to DSBs when single stranded DNA is generated on broken DNA ends. On the other hand, single-strand nicks can result in DSBs when, during replication of damaged DNA, they are encountered by leading-strand DNA polymerases. Therefore these pathways are

frequently activated simultaneously in cells exposed to genotoxic stresses, including ionizing radiation and chemotherapy agents [60].

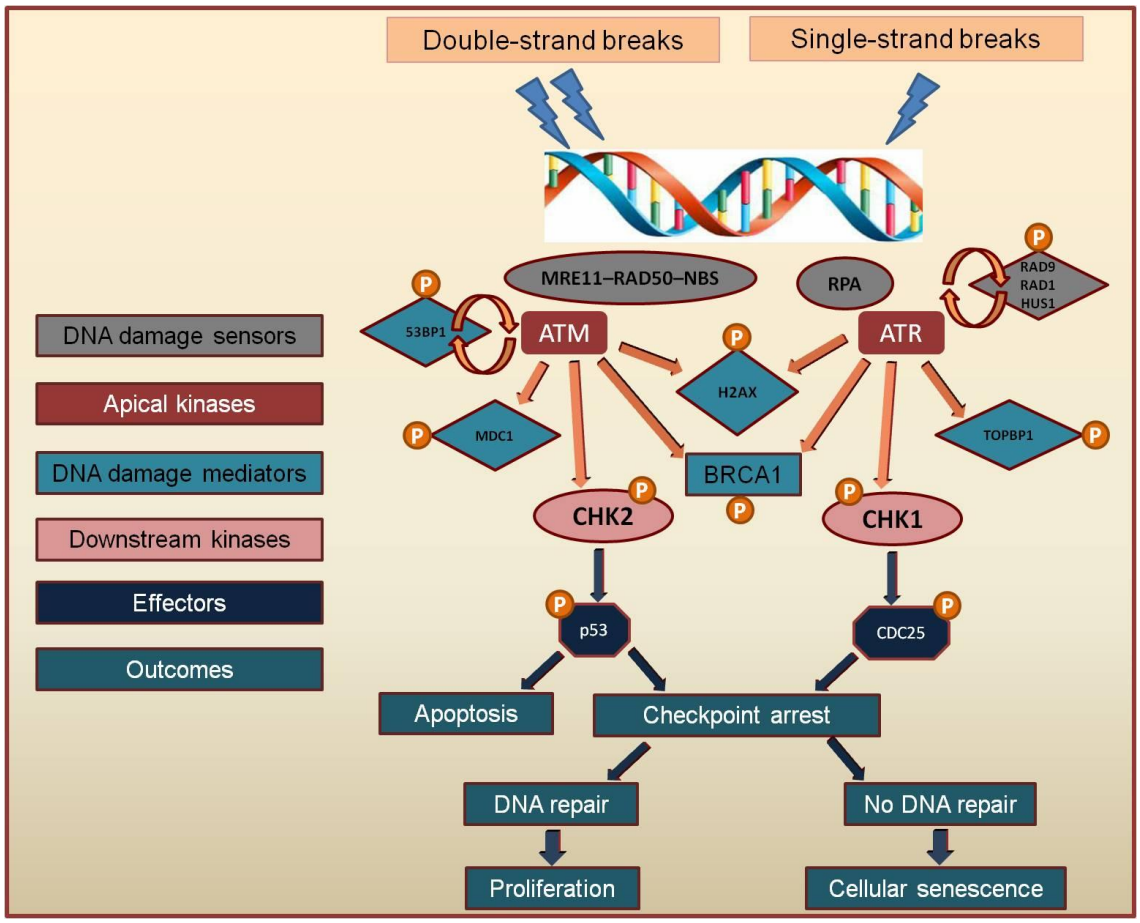

Figure 1.4- The DNA damage response.

Double-strand breaks are recognized by the MRE11–RAD50–NBS1 (MRN) complex and lead to the activation of ataxia–telangiectasia mutated (ATM) and subsequent amplification of the response through the recruitment of other DNA damage response proteins. Activated ATM phosphorylates (P) checkpoint kinases2 (CHK2), which in turn phosphorylates p53 and other substrates. Other forms of DNA damage lead to the generation of single-stranded regions that are detected by replication protein A (RPA) and RAD9–RAD1–HUS1 (9-1-1) complex. This attracts the ataxia–telangiectasia and Rad3-related (ATR) which phosphorylates and is phosphorylated by 9-1-1 complex. ATR activates downstream substrates including checkpoint kinases1 (CHK1). In addition, ATM and ATR phosphorylate the histone variant H2AX on Ser139 (γH2AX). DDR signal amplification is undertaken by damage checkpoint 1 (MDC1), p53-binding protein 1 (53BP1), 9–1–1 complex and topoisomerase-II-binding protein 1 (TOPBP1). Adapted from [4].

Ultimately, when the injury is extremely severe, the DDR may prone cells to apoptosis, a program of cell death that is used to eliminate damaged cells in a controlled manner that minimizes damage and disruption of the neighbour cells [61]. Also, the DDR-initiated

proliferation arrest can be either temporary, allowing cells to repair DNA damage and to resume their cycle, or persistent (cellular senescence), caused by the accumulation of unrepaired DNA lesions that fuel a sustained DDR signalling. It is still unclear what tips the balance towards the pathways that lead to either apoptosis or senescence, but important determinants may include cell type and the intensity, duration and nature of the damage [45, 59].

1.4.1.1 p53 and pRb, two master regulators of senescence

Tumor suppressor genes encode for proteins that impair cell neoplastic transformation. These proteins provide a defence mechanism against oncogenic mutations and induced DNA damage, preventing transformation by forcing cells into apoptosis or cell cycle arrest. Full blown tumor onset depends on the inactivation or deletion of these genes [62].

Consistent with their roles in preventing the proliferation of genetically modified cells, two different tumor suppressor proteins, p53 and retinoblastoma protein (pRb), have been shown to be deeply involved in the onset and maintenance of senescence [2]. Back in 1991 it was shown for the first time that p53 and pRb are two major regulators of senescence, since suppression of both proteins leads to an inhibition of cell cycle arrest allowing cells to bypass senescence [63]. Since then, the role of p53 and pRb in the activation of downstream molecular networks in mouse and human has been subject of extensive research [2].

Experiments with senescent mouse embryonic fibroblasts (MEFs) showed that suppression of p53 is sufficient to escape senescence [64]. On the other hand, although inactivation of pRb alone did not affect senescence, simultaneously with the inactivation of the pRb family proteins p107 and p130 it impaired the cells' capability to perceive the senescence-inducing signal, strongly increasing their proliferation rate [65]. Moreover, acute loss of pRb in senescent cells lead to a reversion of the senescence phenotype [66]. Globally, these results indicate that p53 and pRb are not only necessary for the onset of senescence but are also needed to maintain it. In addition, these evidences suggest a linear model of pathway activation, in which stress-induced p53 activation leads to pRb downstream activation [2].

However, experiments in human fibroblasts suggest that either a linear or parallel activation process can occur [67-69].It is currently accepted that the activation of each tumor suppressor protein can be related to different stimuli and be cell type and species specific [45]. Therefore, pathways downstream of p53 and pRb can be activated independently or simultaneously, cross-regulating each other and mediating senescence onset and maintenance [70, 71].

Different DDR inducers can lead to senescence mainly through the activation of the p53 pathway [72], with the first evidence being dated back to 1984 where ultraviolet (uv) irradiation caused severe genomic stress and increased p53 levels in non-transformed mouse cells [73].

The p53 pathway is regulated at several levels by different proteins, but the most important p53 regulator is the E3 ubiquitin-protein ligase MDM2 (in humans is frequently called HDM2) [74]. MDM2 forms a complex with p53 and inhibits its activity in different ways. MDM2 can bind the transcriptional activation domain of p53 and block its ability to regulate target genes [75, 76], and can also promote the ubiquitylation and subsequent proteosome-mediated degradation of p53, either way inhibiting p53-dependent senescence [77, 78]. Moreover, the *MDM2* gene has a p53 DNA-binding site, making it a direct transcriptional target of p53. Therefore, there is an auto-regulatory negative-feedback loop that regulates the expression of *MDM2* and the activity of p53 [79, 80].

In proliferating cells p53 is kept at basal levels that rapidly increase upon genomic stress. This regulatory mechanism is mainly controlled via the constant action of the MDM2–p53 loop in which excessive p53 activity triggers the production of MDM2 that in turn will promote p53 degradation and extinguish p53 cellular activity [81]. This switch in p53 levels and activity upon stress makes p53 into one of the most important genomic guardians and protectors against cancer progression.

The Rb protein family is composed of pRb, p107 and p130, and is involved in the control of G1-S transition. pRb negatively regulates E2F transcription factors, preventing the activation of genes necessary for G1-S transition and cell cycle progression [82]. Cells lacking pRb show aberrant proliferation and increased genomic instability [83]. It was

recently demonstrated that pRb has a unique and non-redundant function in senescence by regulating a particular subset of E2F targets and preventing DNA replication [83]. pRb specifically represses transcripts associated with replication, while p107 and p130 repress the transcription of DNA replication factors associated with quiescence but not with senescence.

1.4.1.2 The Arf-p53- $p21^{WAF1}$ pathway.

Upstream control of p53 can be mediated by the DDR, via ATM/ATR and CHK2/CHK1 proteins, or by alternative reading frame (Arf) protein [84]. Arf is encoded by the INK4a/Arf locus **(Figure 1.5)**. Formally designated *CDKN2A* and *CDKN2B*, the locus includes three closely linked tumor suppressor genes (*INK4a*, *INK4b*, and *ARF*) that trigger the anti-proliferative activities of both pRb and p53 [85]. *INK4a* and *INK4b* encode for $p16^{INK4a}$ and $p15^{INK4b}$, respectively, and *Arf* encodes for $p14^{Arf}$ ($p19^{Arf}$ in mouse). While $p16^{INK4A}$ is encoded by the α transcript containing exon 1α, exon 2 and exon 3, $p14^{Arf}$ is encoded by the β transcript comprised by exon 1β and exon 2 and 3. These two exons are common to $p16^{INK4A}$ under a different promoter and translated in an alternative reading frame [86].

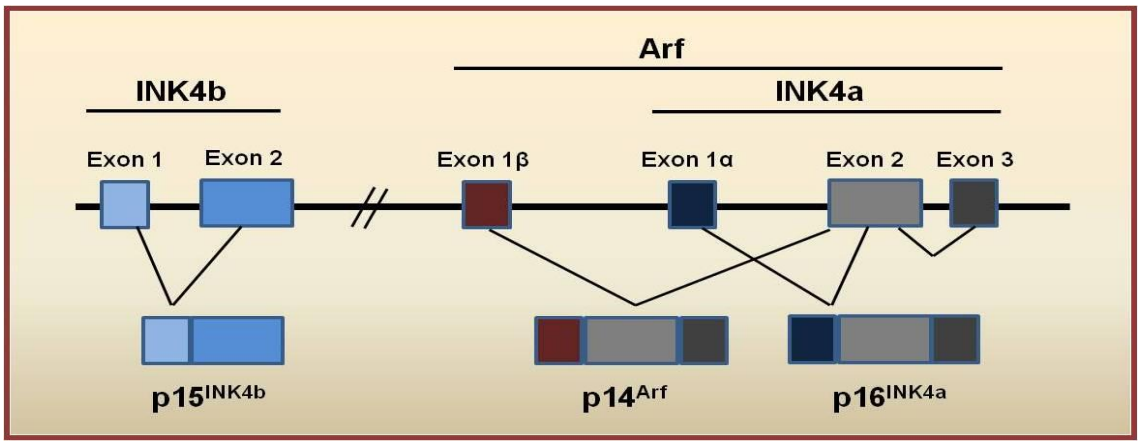

Figure 1.5- Structure of the *INK4a/ARF* and *INK4b* loci.

The INK4b/Arf/INK4a locus includes two different genes. The splicing patterns and encoded proteins are represented. Two distinct promoters transcribes for exons 1α (INK4a) and 1β (Arf) and result in alternatively spliced transcripts that share exons 2 and 3. Although shared, different open reading frames within exon 2 give rise to two distinct protein products. Adapted from [1].

Mice lacking only the 1β exon, expressing only $p16^{INK4a}$, showed high tumor susceptibility, and their phenotype was similar to that of p53 null mice, demonstrating ARF's tumor

suppressor functions [87]. Since p53-deficient cells are resistant to ARF- induced arrest, ARF is considered a positive upstream regulator of p53 [87]. In fact, ARF interferes directly with MDM2 function in the nucleolus, inhibiting the E3 ubiquitin-protein ligase activity of MDM2 and therefore increasing p53 stability and activating the p53-mediated transcriptional program [88, 89].

Despite the fact that several stress signals that can activate the p53 pathway have been identified, those requiring Arf still need to be clarified. It was previously demonstrated that $p19^{Arf}$ knockout (KO) MEFs have impaired DNA damage and microtubule disruption [90]. It was also shown that in normal human fibroblasts Arf expression is induced in response to ionizing radiation, and that endogenous $p14^{Arf}$ can bind directly to p53 independently of HDM2 as an immediate response to DNA damage, suggesting that Arf can also function as a cofactor for p53 transactivation. [91]. In addition, high expression of Arf was also observed in Ras-induced senescent MEFs, and is considered necessary for telomeric and non-telomeric-derived senescence [87, 89]. Although Arf activation seems to be stress dependent, little is known about its physiologic context [2].

In mouse models, Arf inactivation is comparable to p53 inactivation, preventing senescence and reinforcing its role as tumor suppressor [87]. The same is not applied to human cells, where the role of Arf is not so clear [2].

Given the role of INK4a/ARF in modulating activities of pRb and p53, it is not surprising that deletion of the locus is frequently detected in many distinct tumor types [92].

Most genes that are downstream targets of p53 encode for proteins that regulate cell cycle checkpoints, apoptosis and senescence. *CDKN1,* that encodes for cyclin dependent kinases (Cdk) inhibitor protein $p21^{WAF1}$, is directly transactivated by p53 [93]. The $p21^{WAF1}$ protein is a member of the CIP/KIP family of Cdk inhibitors and in the context of senescence, either replicative or stress-induced, is considered one of the most important downstream targets of p53 [94]. All $p21^{WAF1}$'s functions are related to cell cycle inhibition. It not only inhibits cyclin/Cdk complexes, but also blocks proliferating cell nuclear antigen (PCNA), inactivates pRb through proteosome-mediated degradation and inhibits cyclin B1 [95, 96]. Binding of the N-terminal region of $p21^{WAF1}$ to Cdk2 inhibits the formation of the

cyclin E/Cdk2 complexes, thereby impairing pRb phosphorylation. The accumulation of unphosphorylated pRb prevents the release of E2F transcription factors from pRb/E2F complexes, blocking cells in G1 **(Figure 1.6)** [97]. Primarily, $p21^{WAF1}$ activates cell cycle checkpoints in G1 and in G1/S [94]. However, in response to DNA damage, $p21^{WAF1}$ activates the G2/M checkpoint by degradation of cyclin B1, inducing a G2 cell cycle arrest [98].

Nevertheless, senescence is not impaired in MEFs carrying a null mutation in $p21^{WAF1}$, suggesting a non-essential role for $p21^{WAF1}$ or the existence of other mechanisms that compensate for its loss of function [99].

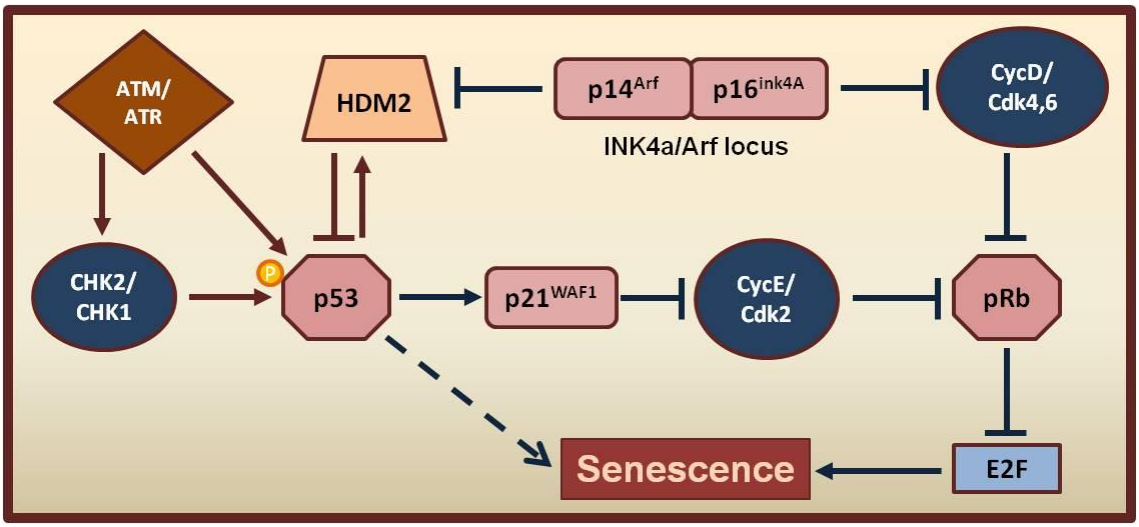

Figure 1.6- Signal transduction pathways mediating senescence.

Sequential activation of the Arf-p53-$p21^{WAF1}$ and $p16^{INK4a}$-pRb signaling cascades. Increased transcriptional activity of p53 is achieved by phosphorylation (P), performed by the ATM/ATR and CHK2/CHK1 proteins, or by the $p14^{Arf}$ product of the INK4a/Arf locus, which sequesters E3 ubiquitin-protein ligase HDM2. By its turn HDM2 regulates p53 activity by a negative feedback loop mechanism. Senescence is triggered by retinoblastoma (pRb) hypophosphorylation and consequent silencing of E2f target genes. pRb is activated either by $p21^{WAF1}$, or by the $p16^{INK4a}$ product trough inhibition of the cyclin E/Cyclin dependent kinase 2 (CycE/Cdk2) and cyclin D/Cyclin dependent kinase 4/6 (CycD/Cdk4/6) complexes, respectively. Adapted from [2].

1.4.1.3 The $p16^{INK4a}$-pRb pathway

$p16^{INK4a}$, also encoded by the INK4a/ARF locus, is a Cdk inhibitor that forms a complex with Cdk4/6, inhibiting the interaction with cyclin D through a conformational alteration.

Abrogation of the activation of Cdk4/6 maintains pRb in the hypophosphorylated active form **(Figure 1.6)** [100].

The $p16^{INK4a}$ promoter is not normally expressed in differentiated tissues. However, it can be induced by stress conditions and was found to be highly expressed in senescent cells [101]. $p16^{INK4a}$ overexpression was first described during replicative senescence [102] and later in response to oncogenic *Ras* activation [57].

Stimuli that trigger a DDR can also activate the $p16^{INK4a}$-pRb pathway, as a secondary response after activation of the p53 pathway [103]. As previously mentioned, the signalling pathway engaged at the onset of senescence can be different according to the stress signal (specific combination and severity), cell type and species. If, on one hand, telomere disruption activates the p53 pathway, on the other hand, other senescence-inducing stimuli, like oncogenic *Ras*, may act primarily through the $p16^{INK4a}$-pRb pathway [67, 104].

$p16^{INK4a}$ KO mice are more prone to develop a variety of spontaneous long latent tumors [105]. In humans, introduction of activated Ha-*ras* into $p16^{INK4a}$ inactivated primary fibroblasts can induce neoplastic transformation [104]. All the evidences point to $p16^{INK4a}$ as a key player in tumor suppression and a mediator of the senescence process.

1.4.2 Senescence-associated heterochromatic foci

Senescent cells suffer a nuclear rearrangement correlated with critical gene expression alterations that will epitomize activation of senescence regulators and silencing of proliferation-associated genes.

As described before, pRb is a crucial effector of senescence and a key component in the irreversibility of the cell cycle arrest. The role of $p16^{INK4a}$-pRb pathway in senescence also involves chromatin remodelling and formation of heterochromatin regions, known as senescence-associated heterochromatic foci (SAHF). At these regions heterochromatin is condensed, modified and transcriptionally silent. SAHF formation appears to be dependent on the integrity of the $p16^{INK4a}$-pRb pathway, in which hypophosphorylated active pRb silences E2F target genes [106].

Initially, histone chaperones like histone repressor A (HIRA) and heterochromatin protein 1-γ (HP1γ) are recruited to specific subnuclear organelles, the acute promyelocytic leukaemia (PML) nuclear bodies. After HIRA's translocation into PML bodies, chromatin condensation occurs, trimethylation of lysine 9 in histone 3 (H3K9me3) accumulates, and HP1 and histone H2A variant (a family of three related proteins, macroH2A1.1, 1.2 and 2) are recruited to SAHF [107, 108].

Proliferation-promoting genes, such as E2F target genes (e.g. cyclin A), are silenced by incorporation into the SAHF, contributing to the irreversibility of the proliferative arrest that characterizes senescence [106]. Senescent cells are, therefore, unable to re-enter the cell cycle, even in a pro-mitogenic environment. These features distinguish senescence from quiescence, a non-proliferative but reversible state [106].

Although senescence can be a stress-response mechanism to prevent proliferation, some senescent cells also become resistant to certain apoptotic signals. For example, senescent human fibroblasts do not undergo ceramide-induced apoptosis, and also become resistant to programmed cell death caused by growth factor deprivation and oxidative stress [109, 110]. In contrast, Fas death receptor activation is able to induce apoptosis in these cells [111]. Nevertheless, the molecular pathways involved in resistance to apoptosis are still poorly understood. Alterations in gene expression may contribute to inhibit, promote or establish apoptosis [112]. Furthermore, evidences show that p53 might preferentially transactivate genes that arrest proliferation, rather than those that trigger apoptosis in senescent fibroblasts [113].

1.4.3 Senescence-associated secretory phenotype

Senescent cells remain metabolically active, acquiring a designated senescence-associated secretory phenotype (SASP), with a characteristic senescence-associated secretome (SAS) composed by several pro-inflammatory cytokines, chemokines, growth factors, and proteases [114].

Persistent DNA damage seems to be required for induction of SASP. In fact, ectopic overexpression of p21 or $p16^{INK4a}$ triggers senescence without SASP, while DNA damage, dysfunctional telomeres, epigenomic disruption, mitogenic signals, oxidative stress, and other senescence-DNA damage inducing stimuli induce a SASP [115, 116].

Moreover, SASP can be different according to the inducing stimulus. Genetic alterations like loss of p53, or activation of *Ras*, that induces senescence by indirect DNA damage due to hipper-replication [117], lead to an accelerated acquisition of a more prominent SASP [118].

The first evidences of alterations in the cell secretome due to senescence were reported in 1991 where microarray analysis revealed a strong inflammatory secretome of fibroblasts undergoing replicative senescence [119].

Currently it is well established that normal and epithelial tumor cells have a SAS after genotoxic-induced senescence [118, 120]. In addition, it was demonstrated *in vivo* that DNA-damaging chemotherapy-induced senescent human prostate cancer cells have a SAS [118].

Using antibody-array technology, a large-scale characterization of SAS from normal fibroblasts, normal epithelial cells, and epithelial tumor cells after genotoxic stress, showed that SAS molecules can be divided into three major categories: soluble signaling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins/extracellular matrix (ECM) components [114, 118]. Overall, these factors can induce mechanisms capable of altering cell fate of neighboring cells and modify the tissue microenvironment. Despite of a substantial overlap in the SAS composition among different cells and tissues, like interleukin (IL)-8, the SAS and its effects seem to be cell and tissue-type specific [114].

The SAS has powerful paracrine effects inducing a variety of cellular responses. With several cytokines and chemokines in SAS, it can contribute to modulate immune responses, targeting the senescent cells for elimination by the immune system, attracting and activating natural killer cells, macrophages and T cells. In addition to an eventual clearance of senescent cells, local immune reactions seem to be stimulated by the SAS, in

order to eliminate oncogene-expressing cells [121, 122]. Recent evidences suggest that SAS molecules can also establish a communication with the surrounding cells in injured tissues promoting the regeneration and/or repair of the tissue lesion. In fact, in two different mouse models of acute liver injury and skin wounding, it was shown that the presence of senescent cells within the damaged tissue was correlated with limited development of fibrosis during tissue repair, contributing to an optimal tissue repair [123, 124].

There are also evidences that suggest an autocrine effect of SAS. In the case of Ras or BRAF-induced senescence in human diploid fibroblasts, the pro-inflammatory cytokines IL-6 and IL-8, and insulin-like growth factor-binding protein 7 (IGFBP7) strengthen the proliferation arrest. In fact, IL-6 is required for both induction and maintenance of oncogene-induced senescence and IGFBP7 has a central role in BRAF-mediated senescence [125, 126].

In contrast to the above described beneficial effects of SAS, several findings suggest that also some detrimental effects can occur. The proliferative rate, migration and invasion of premalignant and malignant cells are enhanced when co-cultivated with senescent fibroblasts or incubated with their conditioned medium [127].

In breast cancer, senescent human fibroblasts can stimulate the proliferation of premalignant and malignant mammary cells *in vitro*, and the formation of tumors in mice [128, 129]. Stromal cells exposed to low-doses of ionizing radiation have been shown to contribute to breast carcinogenesis by disturbing the mammary microenvironment and inducing inappropriate epithelial cell growth in the mammary gland. Part of these alterations may be related to the secretion of multiple matrix metalloproteinases (MMPs) that completely disrupted the mammary ductal morphogenesis as demonstrated in a three-dimensional co-culture system [130]. Moreover, high levels of IL-6 and IL-8 as well as hepatocyte growth factor (HGF), urokinase-plasminogen activator receptor (uPAR) – a serine protease and regulator of the plasminogen activation pathway- and MMPs, secreted by senescent fibroblasts are apparently responsible for enhancing the invasiveness potential of a panel of breast cancer cell lines conferring metastatic

properties on epithelial cells, typical of the epithelial-mesenchymal transition (EMT) [118, 129].

Human prostate stromal cells that undergo senescence secrete a subset of paracrine factors capable of favoring epithelial cells to hyper-proliferate *in vitro*. In fact, fibroblast growth factor 7 (FGF7), HGF, and amphiregulin (AREG) were elevated in the extracellular environment of the senescent prostate fibroblasts and apparently related with the growth-promoting effects [131].

SASP components can also stimulate pro-angiogenic events. Using a xenograft mouse model of breast cancer cells, it was shown that blood vessel density was significantly higher when tumors developed in a senescent microenvironment, and that endothelial cells were more invasive when incubated with conditioned medium from senescent fibroblasts [132].

The complexity of studding the effects of SAS is reflected in the multiple functions of the same SAS component. Whereas IL-6 is required, through an autocrine way, to oncogene-induced senescence, the same senescent cells produce IL-6 that will act as a pro-mitogenic factor through a paracrine fashion [125].

In melanoma, senescent cells secrete several chemokines that may contribute to the emergence of cancer stem cells and therefore contribute to drug resistance, and tumor spreading, as previously reported [133].

Globally, senescence acts as tumor suppressor mechanism, via an irreversible cell cycle arrest. Some SAS factors contribute to the onset of senescence and to stimulate the immune system in a cell non-autonomous way. On the other hand, a senescent microenvironment creates cancer permissive conditions and stimulates cancer cell aggressiveness. In particular, the SAS paracrine effects can induce proliferation and invasion of pre and neoplastic cells, promoting cancer progression. Therefore, according to the physiological context, a senescent cell can be beneficial or detrimental to the neighboring cells. The specific tissue, genetic profile at the time of senescence, and age-related pathologies, like chronic inflammation, are all factors that contribute to this dual role of senescence and may be important key players to clarify this complex paradox. The

challenge is to understand the benefits of senescence in a specific context and to suppress its negative effects.

1.4.4 Markers of cellular senescence

The identification of the cellular state of senescence relies in different markers that can be used in cultured cells and/or in tissues.

Senescent cultured cells exhibit distinct morphological alterations, such as enlarged and flattened cell shape, with an increase in cytoplasmatic granularity and a prominent nucleus - hallmarks of the senescent phenotype (**Figure 1.7**).

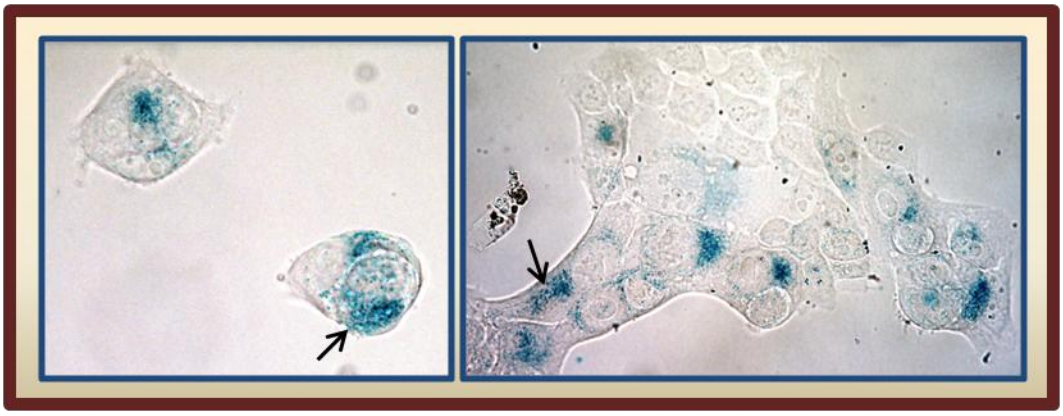

Figure 1.7- Representative images of senescent cells with distinct morphological features *in vitro*.
HCT 116 colon cancer cells become senescent after exposure to 5 μM of 5-FU for seven days. Senescent cells exhibit a characteristic morphology, with enlarged and flattened cell shape and increased granularity. Black arrows indicate the blue cytoplasmatic staining that results from degradation of a chromogenic substrate, 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) by senescence-associated β -galactosidase at pH 6.0. This enzymatic-based detection method is the most widely accepted biomarker of senescence.

However, with some exceptions as reported for large cell change of hepatocytes, the architectural aspects of cohesive tissue organization lead to lack distinctive morphological features of senescent cells present in tissues [53, 134, 135].

The most extensively used biomarker of senescence is the cytochemical detection of β-galactosidase activity at pH 6.0, termed senescence-associated β- galactosidase (SA-β-gal) [136]. The increased activity of this enzyme observed in senescent cells reflects protein overexpression and accumulation at the lysosomas [137]. SA-β-gal is able to cleave the

chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside), leading to the precipitation of an insoluble blue dye visible under a bright field microscope (see **Figure 1.7**) [136]. Activity of SA-β-gal above a threshold level turns it detectable at suboptimal pH 6.0 (pH 4.0 is the optimal), contributing for the specificity of this marker. The increase in SA-β-Gal activity is specific of senescent cells, either triggered by telomere erosion, DNA damage agents, oncogenic signals, or overexpression of tumor suppressors such $p16^{INK4a}$ and ARF [135]. Since it is not observed in pre-senescent or quiescent cells, detection of SA-β-gal activity is considered a reliable marker for detection of senescent cells. [54, 138-141].

Nevertheless, detection of increased activity of SA-β-gal has been also reported in cultured cells under stress conditions, such as serum withdrawal, cell contact inhibition or high confluence, therefore senescent-independent [142]. Moreover, this biomarker cannot be applied to formalin-fixed paraffin-embedded (FFPE) tissues.

Expression of proteins encoded at the *CDKN2A* locus has proved to be a useful marker of senescence in tissues. Increased expression of $p16^{INK4a}$ and Arf has been reported in murine tissues with oncogene-induced senescence, as well as in human malignant tissues [138, 143]. Consistently with the use of Cdk inhibitors to detect senescence, over-expression of $p21^{WAF1}$ was used in human non-alcohol-related fatty liver disease with positive results [144].

γH2AX expression has also been used as a marker of cellular senescence [145]. However, since activated DDR can also induce DNA repair or apoptosis, γH2AX by itself can lead to false positive identification of senescent cells. Proteins associated with SAHF, such as HP1γ and macroH2A histone, have been also used to localize oncogene-induced senescent cells [138, 146].

Up-regulated genes in oncogene-induced senescence, like basic helix–loop–helix transcription factor (*DEC1*) and decoy receptor 2 (*DCR2*), were also candidate markers of cellular senescence [138]. DEC1 and DCR2 are increased in premalignant neoplastic lesions of the skin, lung and pancreas, and highly correlated with the presence of SA-β-gal

activity and SAHF. However, an overexpression of these proteins is not observed in malignant tumors.

Another candidate was lamin B1 [147]. Lamin B1 is decreased in different types of senescent cells, but not in quiescent and proliferating cells, independently of the trigger and signaling pathway involved in senescent onset.

Recently, it was reported that Sudan-Black-B specific stain for lipofuscin can be used as an alternative or complementary method to SA-β-gal activity, in the identification of senescent cells, and suitable for FFPE tissues [148]. Lipofuscin is an autofluorescent lysosomal pigment that is associated with certain processes of ageing and which accumulates during senescence [149].

However, both of these markers are less widely used, partly due to the lack of validated and reproductive protocols especially concerning FFPE samples, requiring further confirmation about the effective identification of senescent cells in an extensive range of samples.

It is clearly evident that until now there is no universal marker of cellular senescence despite all efforts and intensive research over the years. When combined, several markers can be used to identify senescent cells in culture and in tissues. However, there is a lack for specificity, especially for tissues. The use of multiple senescence markers, combined with proliferation indicators like Ki-67 or the incorporation of 5-bromodeoxyuridine (BrdU), which allows the identification of senescent areas with lower levels of proliferation, is still considered to be the best strategy to identify senescent cells [135]. This approach will reinforce an unambiguous detection of a senescent cell.

1.4.5 Significance of cellular senescence in cancer

Oncogenesis and cancer progression comprises several steps, the “hallmarks of cancer” [150]. Genomic instability, oncogenes activation and loss of tumor suppressors are all related events that eventually lead to a scenario of uncontrolled proliferative signaling, apoptosis resistance and replicative immortality. In addition, tumor microenvironment, where a niche of tumor-associated cells will stimulate angiogenesis and create permissive conditions for invasion and dissemination of malignant cells and the ability of tumor cells

to evade the host's immune response, are also important factors leading to full malignancy.

Since cellular senescence induces a state of irreversible cell cycle arrest, it is considered a tumor suppressor mechanism [151]. Evidences that senescence induction can suppress tumorigenesis have been reported in animal models and human cancers.

In mouse mammary epithelial cells genetically manipulated for induction of premature senescence, it was observed suppression in the development of breast cancer, in young female mice exposed to the mouse mammary tumor virus [152]. In a mouse model of lung cancer, a conditional mutation in oncogenic *Ras* induced the development of multiple pre-neoplastic lung adenomas and adenocarcinomas. Senescent cells were observed in adenomas but not in adenocarcinomas [138]. Similar results were observed in animal models of pancreatic cancer and skin papillomas, suggesting that oncogene-induced senescence may help to restrict tumor progression by arresting incipient tumors at a premalignant stage [138].

It was also reported that pre-malignant human nevi and colon adenomas had senescent cells, which were substantially less present in malignant melanomas and colon adenocarcinomas [141, 153].

It is still not entirely clear how pre-malignant lesions progress to full malignancy. One hypothesis is that only a fraction of a heterogeneous population of cells is able to propagate successfully while many undergo apoptosis or senescence [138]. These cells do not fully attenuate the oncogenic signals and are at the origin of the malignant tumors. In the end it will be the balance between proliferative non-senescent and growth arrested cells that will determine the growth rate and tumor formation. Other possibility may imply that cells acquire specific mutations in tumor suppressor genes or suffer epigenetic changes that will enable cells to overcome the proliferative barrier imposed by cellular senescence and progress [154].

Accordingly, it would be expected that neoplastic transformation is related to senescence program inhibition/overcome, nevertheless, it is now broadly described that tumor cells can be induced to undergo senescence upon DNA-damaging stimuli [155].

Furthermore, there are evidences that cellular senescence may even contribute to treatment outcome [54, 156]. However, its effects in the clinical outcome of cancer patients remain controversial due to contradictory results. Data from a mouse model of doxorubicin-treated mammary tumors showed that tumor cells induced by doxorubicin to undergo senescence were apoptosis-resistant and exhibit autocrine/paracrine activity likely contributing to cancer relapse [157]. Moreover, senescent cells were also detected after neoadjuvant CT in patients with malignant pleural mesothelioma or non-small cell lung carcinoma, and correlated with low OS and shorter PFS (p= 0.0098 and p= 0.022, respectively) in the first case, and with a decrease in OS (p= 0.04) in the second [158, 159]. In contrast, CRC patients with senescent cells detected before treatment showed an increased susceptibility to TIS and better chemotherapy response [160]. Elucidation of the specific implications of senescence in the clinical outcome of cancer patients would definitely contribute to treatments improvement and to decrease the side effects of cancer therapy.

Cancer incidence, like several other diseases, increases in with advanced age and the reasons are complex and depending on several factors, including accumulation of mutations in tumor suppressive and tumor promoting pathways and failure of the immune surveillance [116].

Cellular senescence is extensively reported as a common process that links ageing and cancer. Senescent cells are relatively rare in young organisms, but their number increase with age. Inducing factors like replicative exhaustion (telomere shortening), DNA damage (from endogenous and exogenous sources), loss of heterochromatin and inappropriate activation of mitogenic pathways, are more frequent with age [161]. The senescence accumulation is also related with clearance by the immune system. Either because elimination is incomplete (therefore senescent cells gradually accumulate) or because aged individuals generate senescent cells faster than their immune system can handle, senescence has been deeply implicated in ageing [162].

Giving some examples, SA-β-gal positive cells were shown to increase with age in certain human tissues including skin, liver, adipose tissue, skeletal muscle and eye [136, 143, 163].

Senescence comes with age. However, the inverse is also a reality. As expected, the chronic presence of cells that secrete several proteins with potent biological effects significantly alter the tissue structure and the local microenvironment not only contributing to the ageing process but also promoting age-related pathologies.

By depleting the stem and progenitor cells from tissues, cellular senescence compromises tissue regeneration and repair and without the normal tissue turnover, organs start to be functionally compromised [164].

Moreover, SAS factors severally affect vital processes like cell growth and migration as well as tissue architecture by inducing blood vessel formation and differentiation [129]. In addition, potent inflammatory cytokines are SAS components which may be linked to systemic increase of chronic inflammatory reactions known to enhance with age. Chronic inflammation is a hallmark of ageing that initiates or promotes most of the major age-related diseases.

Nevertheless, clear evidences that senescent cells lead to ageing remains circumstantial and we must take in consideration that organisms with germline deficiencies in p53, and consequently with cells that fail to undergo senescence, do not live longer, instead they die prematurely of cancer [165]. If on one hand, the beneficial effects on tumor suppression show promissing unexplored therapeutic targests, on the otherside, the detrimental effects of tissue degeneration must also be take in consideration [166].

Overall, and through SAS, cancer and other age-related pathologies can be promoted by the same mechanism. Attempting to explain how does senescence participates in three apparently opposing processes like tumor suppression, tumor promotion and ageing, several authors support the hypothesis that cellular senescence is an example of the evolutionary theory of antagonistic pleiotropy.

Antagonistic pleiotropy has been used to explain how genetic features can have both beneficial and deleterious effects for species. This theory states that some genes, selected to maintain the health and fitness of young organisms, can have deleterious effects in aged organisms [44].

Thus, DNA damage, telomere erosion or errors in proliferation pathways may cause senescent cells to accumulate, but their influence may become significant and deleterious only later in life when they reach sufficient numbers. The senescence response may have evolved to prevent cancer in most of the life span. However, in organisms that survive longer, the detrimental effects of senescent cells may become apparent [161].

Moreover, mutations accumulate with age. With ageing, there is an increasing probability that senescent cells may occur nearby cells with oncogenic mutations. And despite protecting from cancer in young adults, favorable conditions can be created in order to senescent cells promote the growth and neoplastic progression of the mutant cells [167].

Globally, senescence may benefit benefits organisms early in life by preventing cancer while their accumulation may be detrimental later in life stimulating ageing and age-related diseases like cancer.

1.5 Aim of the thesis

In rectal cancer patients, locoregional relapse and distant metastasis are major events leading to death. Tumor resistance to CRT, which is closely related to tumor relapse and metastatic dissemination, must be elucidated. Tumor cells and tumor-associated host cells, like fibroblasts, may became senescent in response to CT and acquire a SAS that can have deleterious effects on the tissue microenvironment, most likely contributing to tumor invasion and metastasis. **We hypothesize that senescent tumor cells can also be deleterious by affecting neighbor tumor cells through a SAS-mediated paracrine mechanism. Following neoadjuvant CT, cell senescence may become an inducer of cancer relapse.**

For those reasons it is important to elucidate if tumor senescent cells also affect the neighbor cells fate and tumor progression. Understanding these effects will expand the possibilities for cancer therapy and modulation of therapeutic strategies.

Therefore, **the main goals of this thesis were**:

1. To study the effect of CT-induced senescent tumor cells SAS over non-senescent cancer cells, and its spatial range in the rectal cancer tissue microenvironment.
2. To assess if therapy-induced cellular senescence correlates with rectal cancer relapse.

    a) To optimize and validate a biomarker for the detection of senescent cells in formalin-fixed paraffin-embedded (FFPE) tissues.

CHAPTER 2

THERAPY-INDUCED CELLULAR SENESCENCE INCREASES INVASIVENESS AND CHEMOSENSITIVITY IN RECTAL CANCER

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2.1 Abstract

DNA damaging agents and ionizing radiation used in the therapy of human cancers may induce senescence of cancer cells. Senescent cells exhibit a secretory phenotype that can affect cancer cell behavior and, eventually, clinical prognosis.

Here, we show that cultured colon cancer cells induced into senescence by exposure to 5-fluorouracil exhibit a senescence-associated secretome capable of paracrine induction of epithelial-to-mesenchymal transition (EMT) in colon and rectal cancer cell lines, of increased cell invasion *in vitro*, and of increased chemosensitivity to 5-fluorouracil. Importantly, using laser-assisted microdissection we found that in rectal cancer samples from patients treated with neoadjuvant chemo-radiotherapy tumor cell niches enriched for senescent cells bookmark regions of increased expression of EMT-related genes (*Slug, Snail, vimentin*) when compared to nearby senescent-null control regions. Together, these data highlight the complex interplay between therapy-induced cancer cell senescence and two parameters with distinct prognostic value, namely increased chemosensitivity and EMT.

Significance

We provide first-hand, strongly suggestive evidence that senescent cancer cells emerging in the context of neoadjuvant chemoradiotherapy for rectal cancer influence the tumor microenvironment by promoting EMT via short range interactions.

We propose that a combined assessment of senescence and EMT may become a useful prognostic tool after chemoradiotherapy for rectal cancer.

2.2 Introduction

Distant relapse affects about 15 to 20% of patients diagnosed with locally advanced rectal cancer, despite all the therapeutic advances [11, 168]. Whether diagnosed in advanced stage of primary tumor, or at any stage in the presence of positive lymph nodes, the standard of care treatment for rectal cancer patients is neoadjuvant chemotherapy with the thimidylate synthase inhibitor 5-fluorouracil (5-FU) and concomitant radiotherapy (chemoradiotherapy/CRT), followed by surgery [3, 169].

It was previously described that chemotherapy, besides its cytotoxic action, may also induce a cellular state of irreversible proliferative arrest due to severe DNA damage, named therapy-induced senescence (TIS) [42, 43].

Initially considered to be a phenomenon typical of normal somatic cells that lost their ability to divide, thereby named replicative senescence, it is now known that senescence can also be a response mechanism triggered by several factors including oncogenic mutations, oxidative stress, or DNA damaging agents [47, 170].

The effect of cellular senescence in the context of cancer is not completely understood. Cell senescence can play a direct role in tumor growth inhibition since it is an important anti-proliferative mechanism [155]. In lung and breast cancer, detection of cell senescence after neoadjuvant chemotherapy correlated positively with response to treatment [54, 171]. Also in colorectal cancer, patients with sporadic senescent cells detected prior to treatment had increased susceptibility to TIS and better response to adjuvant chemotherapy [160]. However, there is evidence that senescent cells can also exert deleterious effects on the tissue microenvironment [114]. The so called senescence-associated secretory phenotype (SASP) of these cells, which includes the secretion of several pro-inflammatory cytokines, epithelial growth factors and tissue remodeling enzymes, may induce a more aggressive phenotype in non-senescent cancer cells in a paracrine fashion [135]. In fact, data from studies in breast, prostate, and pancreatic cancer, showed that senescent fibroblasts promoted tumor growth and progression by increasing proliferation, invasion, and inducing epithelial-to-mesenchymal transition (EMT) in pre-malignant and malignant cells [118, 128, 129, 131, 172]. It was further

shown that neoadjuvant chemotherapy-induced senescence observed in patients with malignant pleural mesothelioma or lung cancer was potentially associated with a poor outcome [158, 159]. Finally, senescent human prostate tumor cells also have a SASP, raising the question of broader effects of SASPs on tumor behavior [118]. Here, we assessed the effects of the SASP on chemoresistance to 5-FU and induction of EMT *in vitro*, and whether the coupling between cancer cell senescence and EMT induction was recapitulated in clinical samples from patients with rectal cancer subjected to neoadjuvant CRT.

2.3 Material and Methods

Cell lines and human tissue specimens

Human colon carcinoma cell lines HCT 116 and SW48, and the human rectal cancer cell line SW837 were obtained from the American Type Culture Collection (CCL-247, CCL235 and CCL231, respectively). HCT 116 was cultured in McCoy's 5A Modified Medium (Gibco) supplemented with 10 % fetal bovine serum (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 1 % non-essential amino acids (Gibco). SW48 and SW837 were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Gibco). All cell lines were kept at 37°C in 5 % CO_2. Rectal cancer specimens (n=19) were collected during standard of care surgery from patients with rectal cancer who had been treated, or not (controls), with neoadjuvant CRT, included in OCT Compound (Sakura), snapshot frozen in liquid nitrogen within 30 minutes of collection, and preserved at -80°C. This study was approved by the Ethics Commission of Hospital de Santa Maria – CHLN (Lisbon, Portugal), and all patients signed an informed consent.

Induction of cell senescence

$4.0x10^4$ HCT 116 cells were seeded in 60 mm diameter plates and continuously exposed to 5.0 µM 5-FU (Accord Farmacêutica Lta.) or 0.5 µM doxorubicin (Sigma-Aldrich), for 7 days or 4 hours, respectively; media plus/minus (controls) drugs were replaced every 48 hours. After drug removal cells were incubated with fresh medium. Conditioned media were collected 72 hours post-drug release (Senescence-associated secretome/SAS-medium). Culture media conditioned by exponentially growing (non-senescent) cells (non-SAS-medium) were collected 72 hours post seeding. Conditioned media were stored at 4°C and used within 48 hours of storage.

Cellular assays

Detection of SA-β-Gal activity in HCT 116 cells and frozen tissues was performed with the "Senescence Cells Histochemical Staining Kit" (Sigma-Aldrich), according to manufacturer's instructions, followed by counterstaining with nuclear fast red (Sigma-Aldrich) and visualized in a Leica DM2500 bright field microscope (Leica Microsystems).

Cell proliferation was assessed by alamarBlue assay and 5-bromo-2'-deoxyuridine (BrdU) incorporation. AlamarBlue assay (Invitrogen) was performed according to manufacturer's instructions. To determine the effect of conditioned media on cell proliferation HCT 116, SW837 and SW48 cells were plated in 96-well plates ($2.0x10^3$; $1.0x10^4$ and $1.0x10^4$ cells/well, respectively), and incubated for 24 to 72 hours in the presence of SAS-medium or non-SAS-medium. To test for BrdU incorporation cells induced into senescence by 5-FU or untreated control cells were exposed to 10 µM of BrdU for 24 hours or 1 hour, respectively. Cells were then fixed in 3.7 % paraformaldehyde (PFA) for 10 minutes at room temperature (RT); DNA was subsequently depurinated for 30 minutes in 4.0 N HCl, followed by a neutralization step in PBS supplemented with Tris buffer (100 mM; pH8) for another 30 minutes. Cells were incubated with anti-BrdU antibody (1:50; clone BMC 9318, Roche) for 1 hour at 37°C, followed by incubation with anti-mouse Cy3 antibody (1:200**;** Jackson ImmunoResearch). Cover slips were mounted in Vecta-Shield with DAPI (Vector Laboratories), and visualized in a Zeiss Axiovert 200M inverted wide field fluorescence microscope (Carl Zeiss MicroImaging GmbH).

Apoptosis was assessed using the Caspase-Glo® 3/7 Assay (Promega), according to manufacturer's instructions.

Immunofluorescence

Cell lines HCT 116, SW837 and SW48 were seeded on glass cover slips ($2.0x10^5$; $9.0x10^5$ and $9.0x10^5$ cells, respectively) in 60 mm diameter plates and continuously exposed to SAS-medium or non-SAS-medium for 72 hours. After this period the medium was removed, and cells were fixed with 3.7 % PFA for 10 minutes at RT. Cells were permeabilized with PBS/0.5 % Triton X-100 for 10 minutes and then incubated with anti-E-cadherin antibody (1:1000; HECD-1, Invitrogen) for 1 hour at 37°C, followed by incubation with anti-mouse Cy3 antibody (1:200**;** Jackson ImmunoResearch) for 45 minutes at 37°C. Cover slips were mounted in Vecta-Shield with DAPI (Vector Laboratories), and visualized in a Zeiss Axiovert 200M inverted wide field fluorescence microscope (Carl Zeiss MicroImaging GmbH).

Cytokine profiling

Detection and semi-quantification of cytokines present in conditioned media was performed with the "Proteome Profiler™ Array- Human XL Cytokine Array Kit" (R&D Systems) according to the manufacturer's instructions. Briefly, membranes were incubated with 500 µL of conditioned media corresponding to 8 mg/mL of protein, as determined by DC Protein Assay (Bio-Rad). Chemiluminescence detection was done in ChemiDoc MP (Bio-Rad). Pictures were acquired using ImageLab software (version 4.1, Bio-Rad) and imported to ImageJ Software (version 1.48, National Institutes of Health, USA) for image analysis. The mean pixel density in each dot was determined by subtracting the background value, and the average pixel density of duplicate spots was calculated.

RNA isolation and RT-qPCR

Cell lines HCT 116, SW837, and SW48 ($3.0x10^4$; $9.0x10^5$ and $9.0x10^5$ cells, respectively) were seeded in 60 mm diameter plates and continuously exposed to SAS-medium or non-SAS-medium for 72 hours. RNeasy Mini kit (Qiagen) for total RNA isolation and DNase I (Promega) treatment were used as per manufacturer's instructions; RNA concentration and purity was assessed in a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA (1 µg per sample) was reverse- transcribed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's instructions with random hexamer primers. RNA extraction from microdissected tissues was performed using the RNeasy Plus Micro kit (Quiagen) according to manufacturer's instructions. Total RNAs were reverse- transcribed using the RT^2Nano PreAMP cDNA Synthesis kit (SABiosciences) according to the manufacturer's instructions. cDNAs were amplified by semi-quantitative real-time PCR (qPCR) using Power SYBR Green PCR Master Mix (Applied Biosystems) and specific primers in a Rotor Gene 6000 (Corbett, Quiagen). Specific primers were used for the following genes: *CDKN2A* that codes for $p16^{INK4a}$ (PPH00207C), *TYMS* coding for thymidilate synthase (PPH01011B), *IL6* and *IL8* coding for IL-6 (PPH00560C) and IL-8 (PPH00568A) (SABiosciences, Quiagen), respectively; *VIM* for vimentin forward 5'-cgaaaacaccctgcaatctt- 3', vimentin reverse 5'-tcctggatttcctcttcgtg -3'; *FN1* gene encoding for fibronectin, forward 5'- cagtgggagacctcgagaag- 3', fibronectin reverse 5'-

tccctcggaacatcagaaac -3'; *CDKN2A* coding $p21^{Waf1/Cip1}$ forward 5'- ctcagaggaggcgccatgt -3', $p21^{Waf1/Cip1}$ reverse 5'- ccattagcgcatcacagtcg -3'. The genes *SNAI1, SNAI2, ZEB1* and *GAPDH* that encode for Snail, Slug, Zeb1 and GAPDH proteins, respectively were amplified using previously published primers [173]. Relative mRNA expression levels were determined using the $2^{-\Delta ct}$ method and normalized to *GAPDH* housekeeping gene, using the mean value of three replicates.

Invasion assay

The cell invasion assay was performed with the 24-well BD BioCoat™ Tumor Invasion System (BD Biosciences), according to the manufacturer's instructions. Briefly, HCT 116 cells ($5.0x10^4$ cells/ml) were plated in the upper chambers and SAS-medium, or non-SAS-medium (control), was added to the lower chambers. After 24 hours incubation at 37°C with 5% CO_2 the cells in the membrane were stained with 4 µg/ml of Calcein-AM (Calbiochem, Merck Millipore) in Hank's buffered salt solution (Gibco) at 37°C, 5% CO_2 for 1 hr, and fluorescence was quantified in a Infinite200 Multimode reader (Tecan) at 494/517 nm (excitation/emission).

IL-8 quantification

IL-8 in conditioned media was quantified with the Human IL-8/CXCL8 Quantikine ELISA kit (R&D systems), according to manufacturer's instructions.

Laser microdissection

Tumor cells were microdissected in a Laser PALM-Microbeam 4.2 microdissection system (Carl Zeiss MicroImaging GmbH) as previously described [174]. Sequential slides were obtained for the identification of senescent tumor cells (SA-β-Gal activity, as described above) and for laser microdissection.

5-FU chemosensitivity assay

For the determination of the IC50 for 5-FU, HCT 116, SW837 and SW48 cells were seeded in 96-well micro plates with a density of $3.0x10^3$; $2.0x10^4$ and $2.0x10^4$ cells/well, respectively, and incubated for 72 hours (HCT 116 and SW48) or 96 hours (SW837) in

either SAS- or non-SAS-medium, in the presence of different concentrations of 5-FU (0.5 to 500 µM). The alamarBlue assay was performed as described above. IC50 values were determined based on the analysis of dose-response curves using GraphPad Prism software version 5.00 for Windows (GraphPad Software).

Flow cytometry

DNA content was analyzed by flow cytometry. $4.0x10^4$ HCT 116 cells were seeded in 60 mm diameter plates and exposed to SAS-medium or non-SAS-medium for 24 hours. Cells were harvested by trypsinization, fixed with 1% PFA for 3 minutes at RT, and incubated for 10 minutes in 3.7 % PFA/HPEM buffer (Pipes 65 mM, Hepes 30 mM, $MgCl_2$ 2 mM, EGTA 10 mM; pH 6.9)/0.05 % Triton X-100, at RT, for fixation-permeabilization. Cells were washed in PBS and DNA was stained by incubation in propidium iodide (PI) solution (10 µg/ml PI, 0.2 mg/ml RNase A, 0.05 % Triton X-100), for 40 minutes on ice, followed by 40 minutes at 37°C. Samples were analyzed in a BD LSR Fortessa flow cytometer (Becton Dickinson) and cell cycle distribution was analyzed using FlowJo software (Tree Star).

Statistical analysis

Assays were performed in triplicate. Data were analyzed with GraphPad Prism software version 5.00 for Windows (GraphPad Software). Values were expressed as the mean and standard error mean. Differences between groups were analyzed by two-tailed unpaired *t*-test and Fisher's exact test, as appropriate. Non-parametric Mann-Whitney test was used to compare two populations with independent observations. The level of statistical significance was set at $p < 0.05$.

2.4 Results

2.4.1 Low-dose 5-FU induces cellular senescence in HCT 116 colorectal cancer cells

To address the paracrine effects of the secretome from colorectal cancer cells on their non-senescent counterparts, we first induced cellular senescence by exposing HCT 116 colorectal cancer cells to low-dose 5-FU (5.0 µM) for 7 days. At the end of this period we checked for the presence of senescent cells by cytochemical detection of the activity of Senescence-Associated β-galactosidase (SA β-gal). We observed that ~80 % of the cells exposed to 5-FU displayed high levels of SA-β-gal, when compared to control cells **(Figure 2.1A)**.

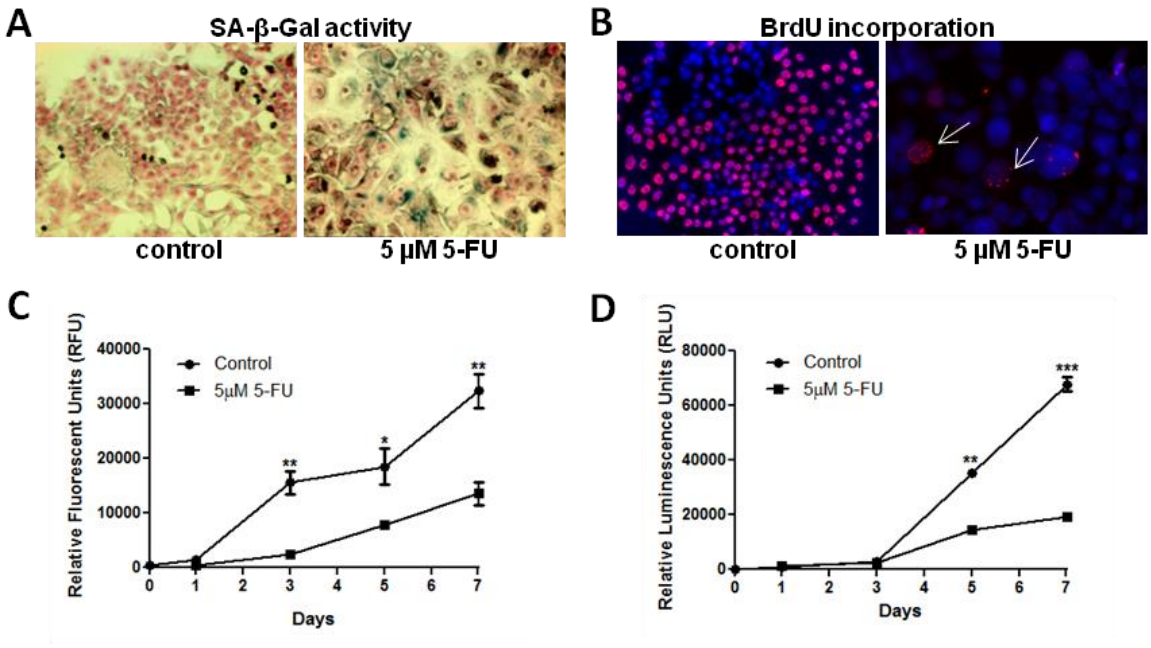


Figure 2.1- Low-dose 5-FU induces cellular senescence in HCT 116 colon cancer cells.

Detection of SA-β-Gal in HCT 116 cells not exposed to 5-FU (controls) or exposed to 5-FU (5 µM) for 7 days. Note the increased staining for SA-β-Gal (blue signal) and enlarged size of 5-FU-treated cells. **B**- HCT 116 cells not treated with 5-FU (controls) or treated with 5 µM of 5-FU for 7 days were incubated with 10 µM BrdU for 1 hour or 24 hours, respectively, and immunostained for BrdU; cells that incorporated BrdU show nuclear staining (red signal; white arrows); nuclei were counterstained with DAPI (blue signal). **C**- Quantification of HCT 116 cells by alamarBlue not exposed to 5-FU (controls) or exposed to 5 µM of 5-FU for 7 days. Analysis of cell growth curves is consistent with ~60 % of 5-FU-treated cells being non-proliferating. **D-** Quantification of apoptosis in HCT 116 cells not exposed (controls) or exposed to 5-FU (5 µM) for 7 days. Caspase-3 and caspase-7 activities were measured by a luminescent assay (Caspase-Glo® 3/7 Assay; cf Materials and Methods). Note that the accumulated apoptosis observed during the induction of senescence by 5-FU is lower than in control cultures not exposed to 5-FU. All evidences suggest a highly senescence enriched HCT 166 cell culture. All determinations were done in triplicate and data are presented as scatter plots of the mean ± SEM. **p* <0.05; ***p* <0.01; ****p* <0.001 based on an unpaired *t*-test.

5-FU-treated cells displayed only residual BrdU incorporation when compared to non-treated controls **(Figure 2.1B)** and reduced proliferation (alamarBlue assay; $p<0.05$ to $p<0.01$) **(Figure 2.1C),** typical features of the senescent state. Measurement of caspase-3 and -7 activities showed that apoptosis contributed little to the reduced cell numbers observed in HCT 116 populations exposed to low doses of 5-FU ($p<0.01$ to $p<0.001$) **(Figure 2.1D)**.

We conclude that low-dose 5-FU induced HCT 116 cells into a senescent state.

2.4.2 The secretome of senescent HCT 116 cells stimulates the proliferation of non-senescent cells

To test the paracrine effects of the senescence-associated secretome (SAS) we first used an array to characterize the cytokine profile of culture media conditioned by HCT 116 cells induced into senescence by 5-FU, hereafter termed “SAS-medium”; controls were provided by culture media conditioned by exponentially growing HCT 116 cells (*cf*, Materials and Methods). This analysis revealed that SAS-media were enriched in IL-8, TGF-α, VEGF, cystatin C, LCN2, MIF, EMMPRIN, and uPAR **(Figure 2.2A** and **Figure 2.2B)** when compared to controls. Since IL-8 is a hallmark of senescence-associated secretomes we further quantified this cytokine by ELISA. The results confirmed a significant increase of IL-8 in SAS-medium ($p<0.05$; Fig. **(Figure 2.2C)**).

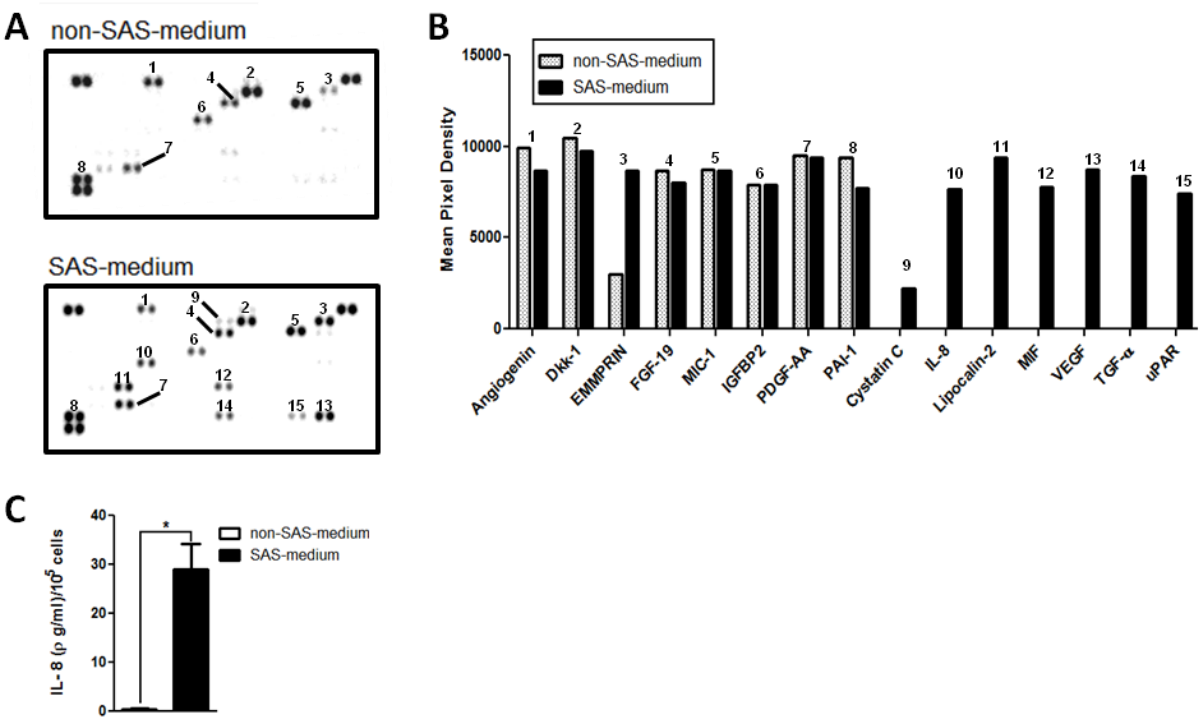


Figure 2.2- Cytokine screening array identifies secreted compounds by 5-FU-induced senescent HCT 116 colon cancer cells.

A- Cytokine screening incubated with either SAS- or non-SAS (controls) conditioned medium obtained as described in Materials and Methods; note that each of the probed cytokines is detected in duplicate (double-spot) in each array**. B-** Shown are profiles of mean spot pixel density created using ImageJ software. **C-** Quantification of IL-8 in media conditioned for 72 hours by either proliferating (non-SAS medium; control) or senescent (SAS-medium) HCT 116 cells. Results are expressed in pg/mL per 10^5 cells, and represent the mean ± SEM of triplicate experiments.

Analyses of cell viability by alamarBlue assay showed that after culturing HCT 116 cells for 24 hours in presence of SAS or non-SAS media (controls), SAS-medium induced a significant increase in cell proliferation ($p<0.05$; **(Figure 2.3)**), in accordance with previously published data for other cell types [128, 129, 131]. To check whether this effect was cell-type specific we also tested the effects of SAS-media on proliferation in a different colon cancer cell line (SW48), and in a rectal cancer cell line (SW837). These results confirmed the early (at 24 h) stimulatory effect of the SAS on the proliferation of these cell lines **(Figure 2.3)**.

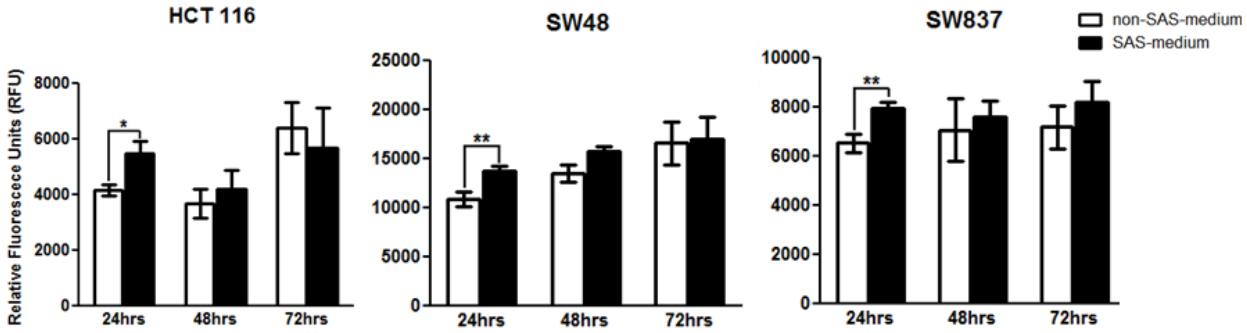


Figure 2.3- The secretome of senescent HCT 116 cells stimulates the proliferation of non-senescent cells.

Quantification of HCT 116, SW48 and SW837 cells by alamarBlue after incubation with conditioned media, either non-SAS-medium (controls) or SAS-medium, for 24, 48 and 72 hours. Represented are ratios between values obtained after and prior to incubation with conditioned media. All determinations were done in triplicate and data are shown as scatter plots of the mean ± SEM. *p <0.05; **p <0.01 based on an unpaired t-test.

These data suggest that the secretome of t HCT 116 cells induced to senesce by exposure 5-FU exerts a positive effect on the proliferation of cycling colorectal and rectal cancer cells.

2.4.3 The secretome of senescent colon cancer cells promotes epithelial-to-mesenchymal transition and increased invasiveness

Next we investigated whether the SAS could affect the epithelial phenotype of proliferating colon and rectal cancer cells. We found that in contrast to incubation with non-SAS control medium, incubation with SAS-medium induced loss of E-cadherin expression, as assessed by immunofluorescence with E-cadherin-specific antibodies **(Figure 2.4A)**. Moreover, our results showed a significant increase ($p<0.05$ to $p<0.001$) in mRNA levels of *Slug*, *ZEB1a*, *Snail*, *fibronectin* and *vimentin* **(Figure 2.4B)**, all hallmarks of EMT, in HCT 116, SW48, and SW837 cells. Also, HCT 116 cells exposed to SAS-medium displayed increased invasiveness compared to control cells exposed to non-SAS conditioned medium ($p<0.001$) **(Figure 2.4C)**.

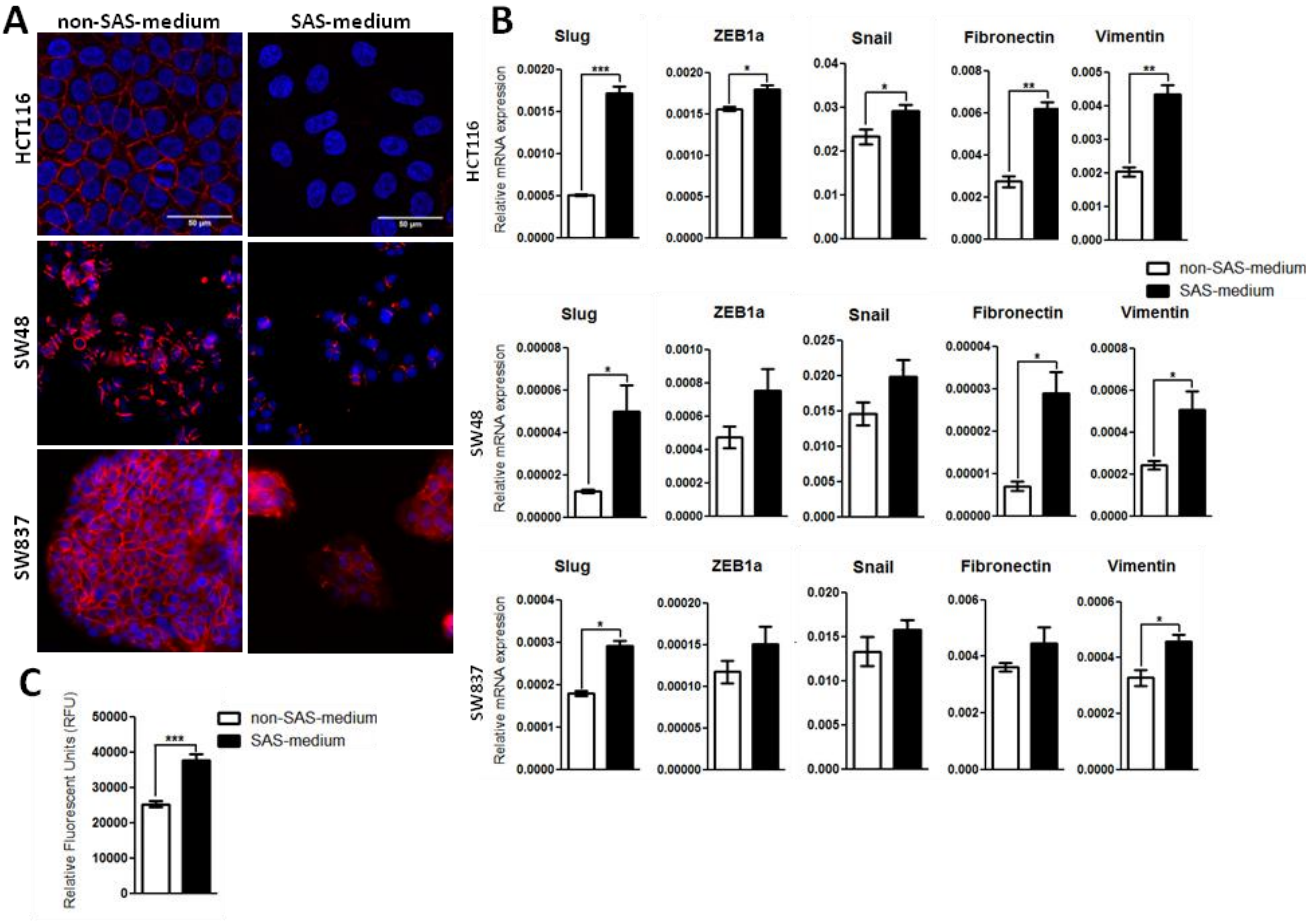
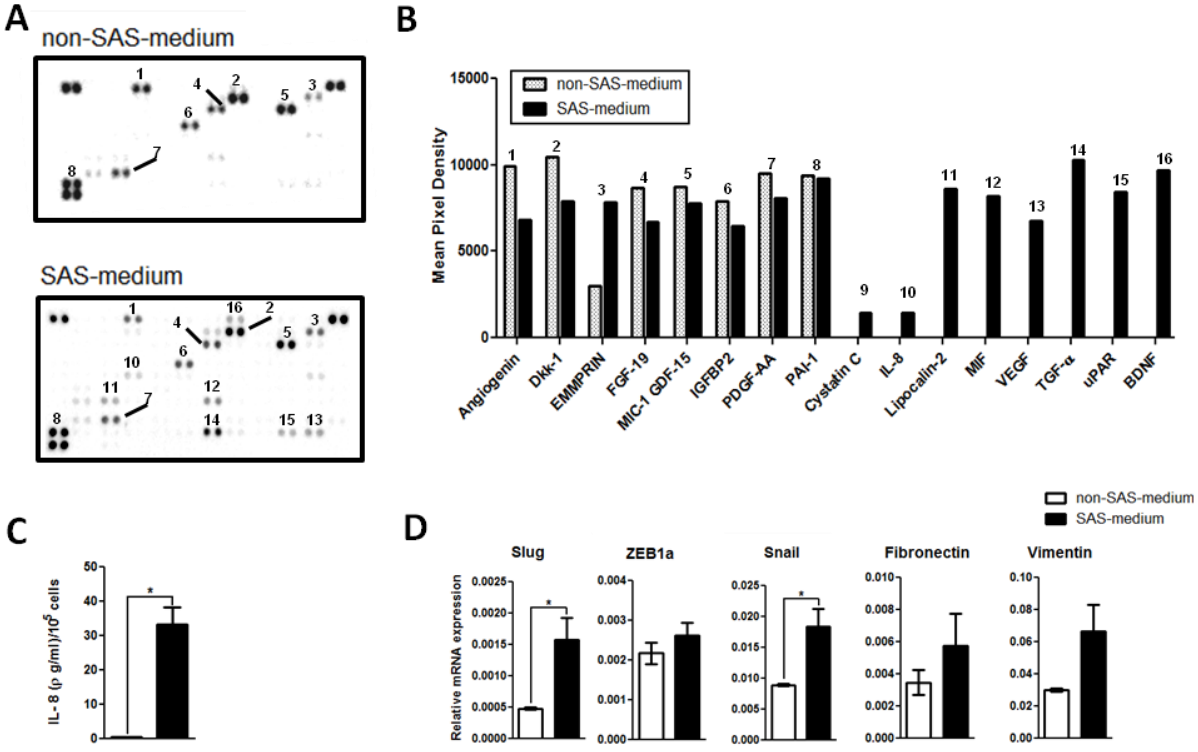


Figure 2.4- The secretome of senescent colon cancer cells induces epithelial-to-mesenchymal transition and increases invasiveness.

A- HCT 116, SW48 and SW837 cells incubated with conditioned media, either non-SAS (control) or SAS-enriched, for 72 hours and stained by immunofluorescence with anti-E-cadherin antibodies (red signal); nuclei were counterstained with DAPI (blue signal). The cellular peripheral E-cadherin signal is either lost or significantly reduced in cells exposed to SAS-medium. **B-** cells exposed to conditioned media as above (non-SAS/control versus SAS-enriched) were analyzed by RT-qPCR for expression of EMT-related genes (*Slug, Zeb1a, Snail, fibronectin* and *vimentin*). Expression of these genes was normalized for *GAPDH* expression. **C-** the chemoattractant properties of conditioned media (non-SAS/control versus SAS-enriched medium) were monitored using a standard invasion assay (cf Materials and Methods). Migrating cells were scored by quantification of Calcein AM-associated fluorescence. All determinations were done in triplicate and data are given as scatter plots of the mean ± SEM. **p* <0.05; ***p* <0.01; ****p* <0.001 based on an unpaired *t*-test.

Finally, we checked whether the observed effects of the SAS on stimulation of EMT and invasive behavior were dependent on the drug used to induce the SAS. To this end, we obtained media conditioned by HCT 116 cells that were induced into senescence by brief exposure to doxorubicin (0.5 µM, 4 hours); of note, this treatment resulted in a proportion of senescent cells (SA-β-gal staining;~ 80%) that was similar to that obtained after exposure to 5-FU (data not shown). Also, within the constraints of the array utilized

here, the cytokine profile of the SAS obtained from doxorubicin-induced senescent HCT 116 cells was similar to that from 5-FU-induced senescent cells **(Supplementary Figure S2.1A, S2.1B** and **S2.1C)**. Similarly, exposure to the doxorubicin-SAS also induced up-regulation of EMT-related genes in proliferating HCT 116 cells **(Supplementary Figure S2.1D)**.



Supplementary Figure S2.1- Cytokine screening array and EMT induction by SAS-medium obtained from doxorubicin-induced senescent HCT 116 colon cancer cells.

A-Cytokine screening arrays incubated with either SAS- or non-SAS (controls) media; SAS-media were conditioned by HCT 116 cells induced into senescence by doxorubicin; each of the probed cytokines is detected in duplicate. **B-** Profiles of mean spot pixel density were created using ImageJ software. **C-** SAS-medium conditioned by senescent HCT 116 cells (doxorubicin-induced senescence) is enriched in IL-8 relative to control (non-SAS) medium. Quantifications were performed by ELISA and are expressed in pg/mL per 10^5 cells. **D-** expression levels of mRNAs from EMT-related genes in proliferating HCT 116 cells incubated for 72 hours with either SAS-medium or non-SAS-medium (controls). Gene expression was assessed by RT-qPCR, and normalized to *GAPDH* expression. All determinations were done in triplicate and data are presented as scatter plots of the mean ± SEM. **p* <0.05 based on an unpaired *t*-test.

In sum, these data show that in presence of senescence-specific secretomes colon and rectal cancer cells are induced into EMT and acquire increased invasiveness.

2.4.4 The secretome of senescent colon cancer cells increases the chemosensitivity to 5-FU

Given the remarkable effects of the SAS in the modulation of phenotypes displayed by HCT 116 and SW48 colon cancer cells, and SW837 rectal cancer cells, we asked whether this might be reflected in a modified response to chemotherapeutic agents, namely 5-FU. To address this issue, we estimated the IC50 for 5-FU in HCT 116, SW837 and SW48 cells exposed for 72 hours to different concentrations of 5-FU (0.5 to 500 µM) diluted in either SAS-medium or non-SAS (control) medium. Analyses of cell viability by alamarBlue assay showed an increased chemosensitivity to 5-FU with significant reduction in IC510 values for each cell line (p=0.0008, p=0.045, p=0.0119, respectively) **(Figure 2.5A)**.

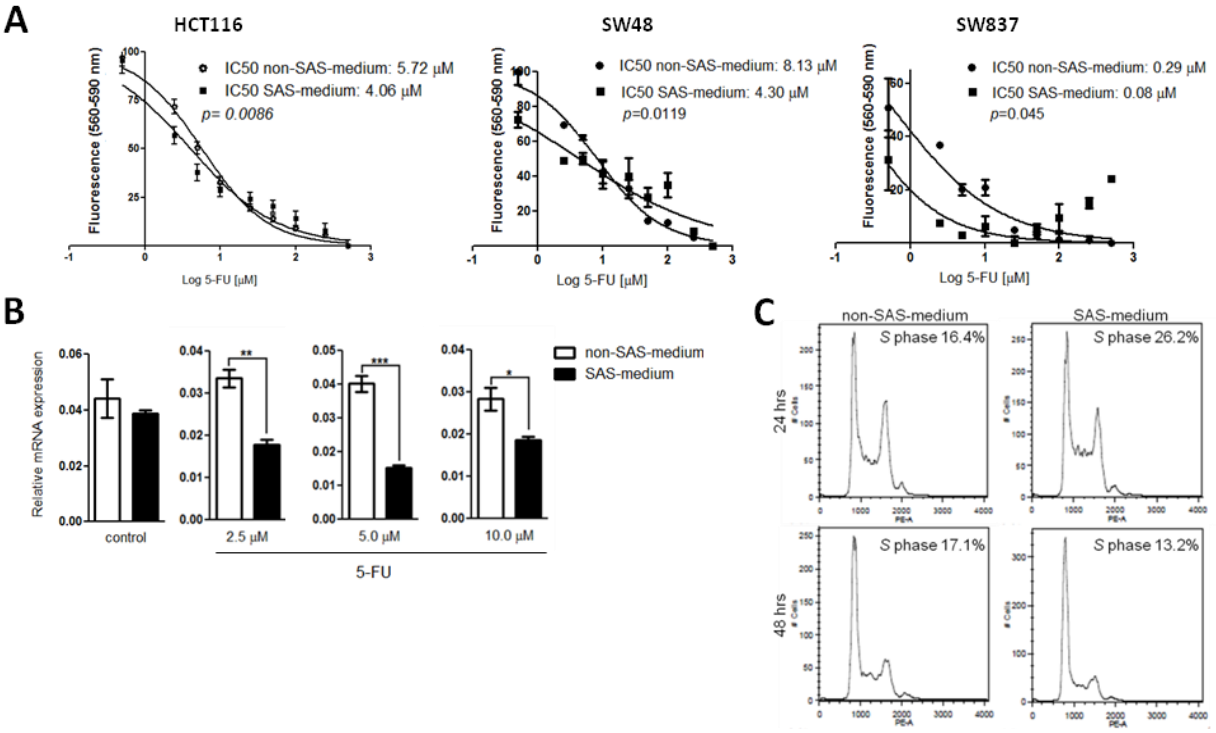
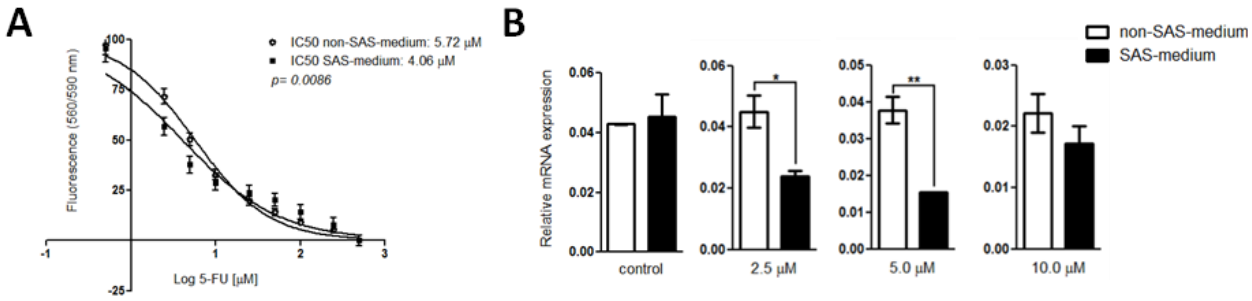


Figure 2.5- The secretome of senescent colon cancer cells increases the chemosensitivity of proliferating cells to 5-FU.

A- Survival of HCT 116, SW837 and SW48 cells cultured in either SAS-medium or non-SAS-medium (controls) and exposed to different concentrations of 5-FU (0.5 to 500 µM; 72 hours for HCT 116 and SW48 cells and 96 hours for SW837 cells). Cell survival correlates positively with fluorescence emitted at 590 nm wavelength (alamarBlue assay; cf Materials and Methods). Curve fitting and IC50 determination were performed using GraphPad Prism software. Data were obtained in triplicate and are represented in the graph by the mean ± SEM. The IC50 dose of 5-FU decreased significantly in presence of the senescence-associated secretome (3.77; 8.13 and 0.29 µM) when compared to controls (5.72; 4.30 and 0.08 µM, respectively). **B-** Levels of thymidylate synthase (TS) mRNA in HCT 116 cells treated with 2.5, 5.0 and 10.0 µM of 5-FU (72 hours) in SAS-medium or non-SAS-medium (controls). *TS* expression was measured by RT-qPCR and normalized to *GAPDH* expression. All determinations were done in triplicate and data are given as scatter plots of the mean ± SEM. *p <0.05; **p <0.01; ***p <0.001 based on an unpaired *t*-test. **C-** HCT 116 cells incubated with SAS-medium or non-SAS-medium (controls) for 24 and 48 hours were analyzed by flow cytometry after DNA staining with propidium iodide. Histograms depict relative distributions across the cell cycle; highlighted is the percentage of cells in S phase as determined using FlowJo software.

Comparable results were obtained when the SAS was induced by doxorubicin; again, in presence of this latter SAS-medium the IC50 for 5-FU of HCT 116 cells was reduced from 5.72 µM (non-SAS controls) to 4.06 µM (p=0.0086) **(Supplementary Figure S2.2A)**.



Supplementary Figure S2.2- The secretome from colon cancer cells induced into senescence by doxorubicin increases chemosensitivity of proliferating HCT cells to 5-FU.

A- Exposure of HCT 116 cells to increasing doses of 5-FU (+/- SAS-medium), quantitation of cell proliferation by alamarBlue, curve fitting and graphical representation, and IC50 determination were as described previously in Figure 3. The IC50 of 5-FU decreased significantly in presence of the senescence-associated secretome (SAS-medium; 4.06 µM) when compared to controls (5.72 µM). **B-** levels of thymidylate synthase (*TS*) mRNA in HCT 116 cells treated with 2.5, 5.0 and 10.0 µM of 5-FU (72 hours; +/- SAS-medium) as in Figure 3. *TS* expression was measured by RT-qPCR and normalized to *GAPDH* expression. Note the lower levels of *TS*-specific mRNA in cells exposed to SAS-medium. All determinations were done in triplicate and data are shown as scatter plots of the mean ± SEM. **p*<0.05; ***p*<0.01 based on an unpaired *t*-test.

Since the response to 5-FU critically depends on the cellular levels of thymidylate synthase (TS), we next tested whether the observed SAS-induced chemosensitivity to 5-FU could be related to a lower expression of this enzyme in presence of the SAS. Quantitative RT-PCR data showed that exposure of HCT 116 cells to SAS-medium or 5-FU separately did not significantly affect the expression of TS mRNA **(Figure 2.5B)**. However, the levels of this mRNA were significantly reduced when exposure to 5-FU was in presence of SAS-medium, but not in presence of control (non-SAS) medium ($p<0.05$ to $p<0.001$) (Fig. 5B). Importantly, similar results were obtained when the SAS-medium was obtained from doxorubicin-treated cells ($p<0.05$ to $p<0.01$) **(Supplementary Figure S2.2B)**.

These data strongly suggest that the SAS cooperates with 5-FU to reduce TS mRNA levels.

Since the SAS significantly increased cell proliferation after 24 hours of incubation, and also altered sensitivity to 5-FU, an S phase-specific drug, we asked whether the SAS

promoted any alteration in the cell cycle. To test this hypothesis, HCT 116 cells were cultured in either SAS-medium or else in control (non-SAS) medium for 24 hours, and analyzed by flow cytometry after staining the DNA with propidium iodide. This analysis revealed an augmented proportion of cells in S phase in presence of the SAS (26.2% *versus* 16.4% for control medium) **(Figure 2.5C)**, consistent with the SAS accelerating cell division in particular the entrance into S phase.

Taken together these results show that SASs increase the sensitivity of proliferating colon and rectal cancer cells to 5-FU, and strongly suggest that this stems from the SASs promoting a higher proportion of cells in S phase where sensitivity to 5-FU is highest.

2.4.5 Neoadjuvant chemotherapy promotes emergence of senescence and EMT in human rectal cancers

The previous data highlighted a relevant set of paracrine effects exerted by the SAS on target proliferating colon cancer cells, namely induction of EMT. We then wondered whether these effects might be of more broad clinical relevance, and thus observable in tumors from rectal cancer patients.

We selected as model system rectal cancer samples collected at the time of surgery from patients that had received, or not, neoadjuvant chemoradiotherapy (CRT). Although these samples do not allow us to observe the impact of CRT in samples from the same patient, we were able to compare tumor samples from different patients prior to, and after CRT treatment. Therefore, patients were divided in two different groups according to neoadjuvant treatment **(Table 2.1)**. These two groups were similar concerning gender distribution, mean of ages, pathological primary tumor classification (pT) and regional lymph node (RLN) involvement (p=0.3047, p=0.7553, p=0.3047 and p=0.6332, respectively).

Table 2.1- Patient and tumor characteristics

	Neoadjuvant CRT		
	Yes (n=7)	No (n=12)	*p* value
Male (%)	57	83	0.3047[1]
Age (y), median (range)	70.8 (50-84)	68.6 (43-89)	0.7553[2]
pT classification* (%)			0.3047[1]
pT2	43	17	
pT3	57	83	
RLN involvement (%)	71	50	0.6332[1]

*Staging according to *AJCC Cancer Staging Manual*, 7th ed. New York, Springer, 2010
[1]- Fisher's exact test [2]- Student's *t* test
Abbreviations: **pT**- pathological primary tumor; **RLN**- regional lymph nodes; **CRT**- chemoradiotherapy

In these samples we tested whether CRT induced cellular senescence in tumor cells and, whether proximity to senescent cancer cells correlated with induction of EMT. To this end, frozen rectal cancer samples from patients who were either subjected to CRT (n=7) or not (controls; n=12) were sectioned, and randomly chosen clusters of cancer cells were laser-microdissected free of stromal components for quantitative analysis of specific mRNAs by RT-qPCR.

We initially tested for expression of genes that strongly correlate with the senescent state, namely $p21^{Waf1/Cip1}$, $p16^{INK4a}$, and *IL-8*. Both $p21^{Waf1/Cip1}$ and *IL-8* were found to be up-regulated in the group of patients that received neoadjuvant CRT ($p<0.05$) (Fig. 6A) compared to control (non-CRT) samples, suggesting that CRT induced cellular senescence in these tumors. Next, we analyzed the expression of the EMT-related genes, *Snail*, *Slug* and *vimentin* in the same samples and found these genes to be significantly up-regulated in tumors from patients treated with neoadjuvant CRT ($p<0.05$) **(Figure 2.6A)**.

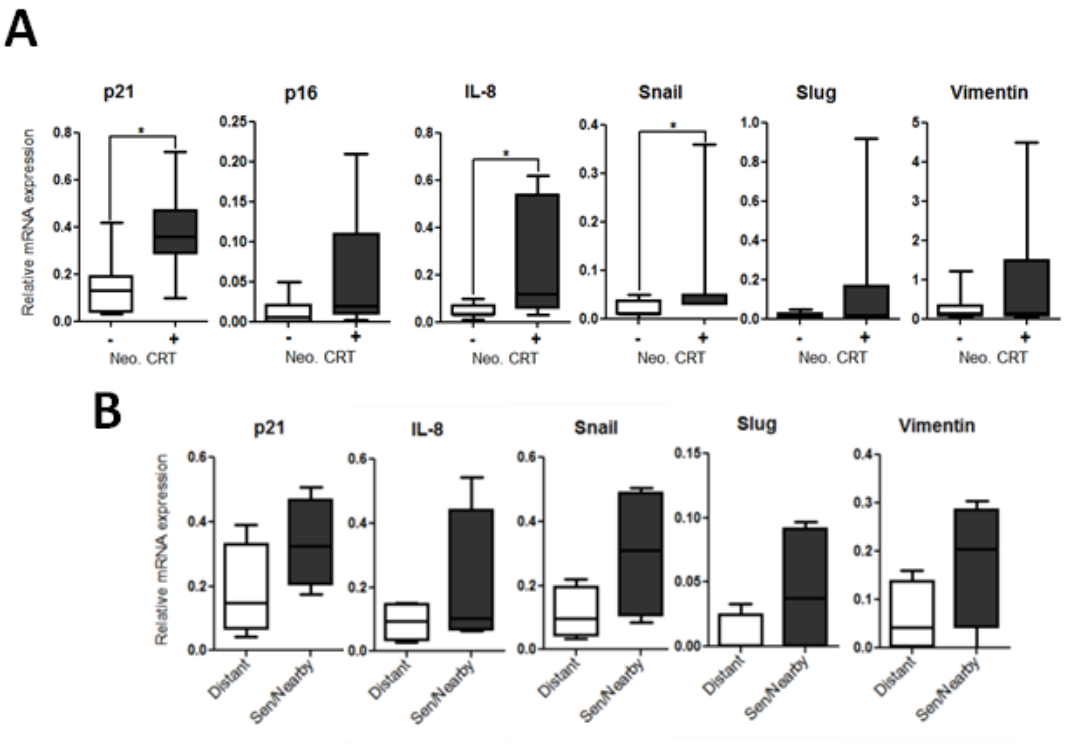
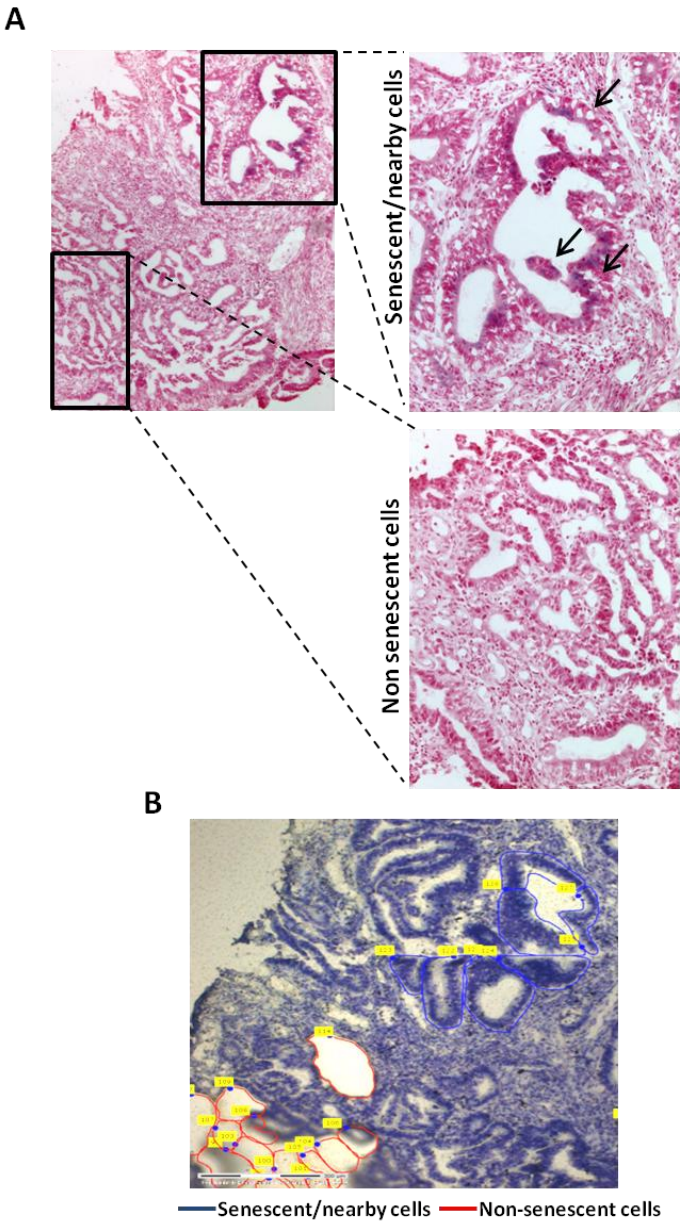


Figure 2.6- Neoadjuvant chemotherapy promotes emergence of senescence and EMT in human rectal cancers.

A- Total RNA was extracted from microdissected clusters of cancer cells from human rectal cancer samples obtained from patients that either received neoadjuvant chemo-radiotherapy (Neo.CRT+; n=7) or not (Neo.CRT-/controls; n=12) prior to surgery. Specified mRNAs were quantified by RT-qPCR and normalized to *GAPDH* expression. **B-** in rectal cancer samples from patients subjected to neoadjuvant chemoradiotherapy (n=4) cancer cell clusters enriched in senescent cells, and clusters locating nearby (>700 µm) and certified as devoid of senescent cells (controls), were laser-microdissected and used for quantification of specific mRNAs by RT-qPCR, as above. Note the increase in both senescence (*$p21^{Waf1/Cip1}$, IL-8*) and EMT markers (*Snail, Slug, vimentin*) in tumor areas enriched for senescent cells. Whiskers represent the minimum and maximum across the data. **p* <0.05 based on a Mann–Whitney *U* test.

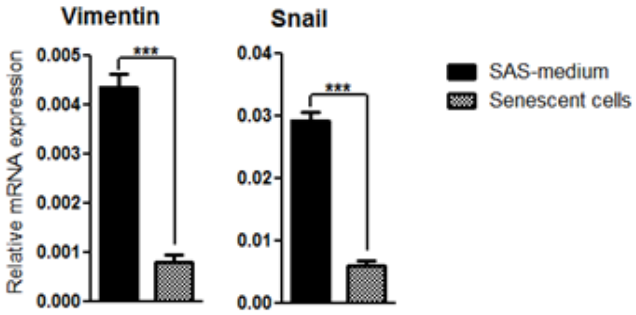
Subsequently, in samples from patients that had received neoadjuvant CRT we obtained serial sections in which every other section was stained for SA-β-Gal and used to directly assess the presence of senescent cells in the contiguous sections (stained with cresyl violet). Staining for SA-β-Gal was detected in discrete regions of tumor tissue, and cancer cell clusters enriched for senescent cells were identified and selected (Supplementary Fig. S3A). These clusters, together with nearby clusters (>700 µm) in which senescent cells were absent (controls), were laser microdissected and used for the analysis of expression of *$p21^{Waf1/Cip1}$* and *IL*-8, and of the EMT-related genes *Snail, Slug*, and *vimentin* by RT-qPCR **(Supplementary Figure S2.3B)**.



Supplementary Figure S2.3- Representative sequential frozen sections of human rectal cancer tissue selected for isolation of senescent-positive and senescent-negative epithelial cell populations by laser microdissection.

A- Representative sequential frozen sections of human rectal cancer tissue selected for isolation of senescent-positive and senescent-negative epithelial cell populations by laser microdissection. A, section stained for SA-β-Gal activity to identify clusters of cancer cells harboring senescent cells (blue staining; upper inset, left; arrows, top right) and nearby clusters devoid of SA-β-Gal staining (lower inset, left; bottom right); counterstaining was with nuclear fast red. **B-** Contiguous section stained with cresyl violet in which areas selected for microdissection are delineated (senescent-positive, blue lines; senescent-negative, already laser-ablated, red lines) Magnification, x100 (A, *left*) and x200 (A, *right*; B).

As expected, *p21*$^{Waf1/Cip1}$ and *IL*-8 were up-regulated in tumor cell clusters enriched in senescent cells **(Figure 2.6B)**. More importantly, *Snail, Slug*, and *vimentin* also showed increased expression in these same tumor cell clusters, indicating that senescence and EMT co-occurred within the same microenvironment **(Figure 2.6B)**. It is unlikely that senescent cells themselves are the source of increased expression of genes related to EMT since this is not part of the drug-induced senescent phenotype. In fact, we found that expression of *Snail* and *vimentin* was significantly lower in HCT 116 cells that reached senescence by exposure to 5-FU compared to HCT 116 cells induced into EMT ($p<0.001$) **(Supplementary Figure S2.4)**.



Supplementary Figure S2.4- EMT-related genes are poorly expressed in senescent cells.

Expression of *vimentin* and *Snail* were assessed by RT-qPCR in HCT 116 cells, either senescent or induced into EMT by exposure to SAS-enriched medium. All determinations were done in triplicate and data are given as scatter plots of the mean ± SEM. ****p*<0.001 based on an unpaired *t*-test.

2.5 Discussion

There is good evidence that the tumor microenvironment has a role in cancer progression [175]. Recently, it was shown that in rectal cancer patients receiving neoadjuvant therapy the patterns of gene expression of cancer-associated fibroblasts provided predictive power for distant recurrence and prognosis, supporting a role for the microenvironment in rectal cancer behavior [176]. Also, treatment of cancer patients with DNA-damaging agents, both in neoadjuvant and curative settings, was shown to induce epithelial cancer cells into accelerated forms of senescence [135]. Senescent cells derived from either non-transformed or cancer cells are associated with senescence-specific secretomes, the SASs [118]. Despite the powerful paracrine effects exerted by SASs *in vitro* [118, 128, 129, 131, 172], it has remained elusive to what extent senescent cancer cells present within tumors influence the microenvironment and, eventually, cancer progression [158-160].

We initially interrogated the cytokine composition of the SAS from colon cancer cells (HCT 116) induced to senesce by exposure to either 5-FU, which targets cells in S phase and interferes with DNA repair, or to doxorubicin, a DNA-damaging drug. We presented evidence that senescent colon cancer cells are able to consistently secrete several cytokines to high levels (*eg*, IL-8), irrespectively of whether senescence was induced by 5-FU or doxorubicin. In addition, we have provided evidence that CD147, cystatin C, LCN2 and TGF-α are also SAS-related molecules, for the first time.

As previously described for other cell types ([118, 129, 172], in two different colon cancer cell lines (HCT 116 and SW48), and in one rectal cancer cell line (SW837), the paracrine effects of the SAS comprised increased proliferation and invasiveness, as well as induction of EMT, all of which correlate with aggressive cell behavior.

Interestingly, exposure of colon and rectal cancer cells to SASs enhanced the sensitivity to 5-FU. Chemosensitivity to 5-FU is known to depend, to a large extent, on cellular levels of thymidylate synthase (TS) [33]. Here, we showed that SASs further promote the reduction of TS levels prompted by 5-FU, thus providing a plausible explanation for the increased sensitivity of the three different cell lines to 5-FU in presence of SASs. Similar results regarding the decrease of TS expression levels could also be observed when HCT 116 cells

were in presence of SAS obtained from doxorubicin-treated cells, suggesting an increase in the sensitivity to 5-FU regardless the senescence-inducing stimuli. Since EMT has been correlated with increased resistance to several anti-cancer drugs [177], whether the effect of the SASs shown here in promoting chemosensitivity to 5-FU is restricted to this drug or else extensive to other cancer-specific drugs warrants further investigation. Moreover, prospective studies shall clarify whether the reduction observed in the IC50 doses of 5-FU are of clinical relevance.

Altogether, these data highlighted that the secretomes from senescent colon cancer cells may elicit concurrent phenomena with antagonistic prognostic value, namely EMT and increased invasiveness versus increased chemosensitivity.

As mentioned, robust induction of EMT by SASs has been previously reported [118, 129], and was shown in this work to occur in cultured HCT 116 cancer cells in response to SASs derived from their senescent counterparts. Our data also showed that, in contrast to cells undergoing EMT, senescent cells express relatively low mRNA levels of EMT markers such as *Snail* and *vimentin*, thus allowing distinction between these two cellular outcomes by gene expression criteria. Given the consistent induction of EMT by SASs, and the known relevance of EMT in cancer behavior, we addressed whether the paracrine effects of senescent cells, via the SAS, on EMT induction seen *in vitro* might be recapitulated *in vivo*, in the clinical setting. Our data showed that in sections of tumors obtained from patients with rectal cancer the senescent cells, identified by their strong staining for SA-β-Gal, were present within discrete clusters of cancer cells. Laser microdissection of these clusters and of nearby ones devoid of senescent cells was performed and followed by RT-qPCR analysis of senescence and EMT markers, $p21^{Waf1/Cip1}$and *IL-8*, and *Snail, Slug* and *vimentin*, respectively. This showed that these genetic markers were co-expressed at significantly higher levels in the cancer cell clusters enriched for senescent cells relative to nearby ones (>700 μm) devoid of them. These data strongly suggest co-occurrence of senescence and EMT within the same cell clusters. They are also consistent with the influence of senescent cancer cells on induction of EMT being relatively short range, not affecting nearby niches of cancer cells. What restricts this influence remains to be established.

Tumor recurrence after preoperative chemoradioterapy followed by surgery with curative intent remains a major problem to successful cancer treatment affecting 15 to 20 % of patients diagnosed with locally advanced rectal cancer [11, 168]. Tumor regression after neoadjuvant chemoradioterapy has been used to evaluate treatment response and prognosis [21, 22]. However, results of neoadjuvant therapy can range from lack of effectiveness to complete pathologic remission. Clearly, the identification of novel predictive biomarkers of disease progression is crucial to improve adjuvant therapeutics and follow-up strategies.

Future studies shall tell whether after neoadjuvant therapy for rectal cancer the presence of senescent cells, and EMT-related changes in their microenvironment, add prognostic power on cancer recurrence and patient survival.

CHAPTER 3

THE IMPACT OF DRUG-INDUCED CELLULAR SENESCENCE IN RECTAL CANCER RELAPSE: A RETROSPECTIVE STUDY

3.1 Introduction

In advanced rectal cancer (T3/T4 and/or lymph node involvement), despite the significant improvement in local control and radiosensitivity, achieved with neoadjuvant CRT when compared with adjuvant (post-surgery) treatments, disease progression often occurs [11, 168].

CT drugs are not always cytotoxic. In fact, tumor cells may undergo cellular senescence induced by CT drugs, radiation, or other DNA damaging agents [54, 170]. This is termed stress-induced or premature senescence, a different concept than replicative senescence **(Chapter 1)** [47, 55, 57].

We have previously shown that cultured colon cancer cells induced to senesce by exposure to 5-FU (but also doxorubicin) have a specific secretome able to paracrinally induce EMT and increased invasiveness in neighbor proliferating tumor cells, but also able to increase sensitivity to 5-FU coupled to reduced expression of TS enzyme. This latter effect was unexpected since typically EMT connotes increased resistance to anti-cancer drugs. Most importantly, the effects of senescent cancer cells on EMT appear to be recapitulated in clinical samples from rectal cancer patients subjected to neoadjuvant CRT. Cellular senescence co-occurred with cells undergoing EMT in discrete niches, but not in nearby cancer cell niches. This provides first-hand evidence that senescent cancer cells influence the tumor microenvironment by promoting EMT via short range interactions. These findings highlight the importance of assessing cancer cell senescence while searching for new prognostic factors after neoadjuvant CRT in the rectal cancer setting.

With the objective to establish a correlation between the presence of senescent tumor cells and local recurrence and distant metastasis in rectal cancer patients, we performed a retrospective study enrolling 35 patients diagnosed with rectal cancer and submitted to neoadjuvant chemotherapy. For this purpose, we first addressed the use of immunohistochemical (IHC) detection of SA-β-gal in FFPE tissues as a reliable marker to identify senescent cells.

When observed *in vitro*, cell senescence is generally accompanied by morphological changes. In particular cases, some features could vary based on the triggering factor, nevertheless senescent cells usually adopt a large and flat morphology, become multinucleated and show increased granularity. Importantly and despite some exceptions, these characteristics are generally not present *in vivo* due to architectural features of the tissues [53, 134, 135].

The only validated biomarker of cellular senescence is the increased activity of the lysosomal protein SA-β-gal at pH 6.0, reflecting a protein overexpression and accumulation of the lysosomal endogenous protein content [136, 137, 178]. There are several studies reporting other potential senescence biomarkers (e.g. γH2AX, DEC1, DCR2, lipofuscin) [138, 145, 148], but the detection of SA-β-gal activity is still the only consensual biomarker of senescence both *in vitro* and *in vivo* [135, 179]. This fact limits the senescence-related studies to the use of fresh frozen tissues. Therefore, it is crucial to validate universal biomarkers for the detection of senescent cells in FFPE tissues.

Although we observed a positive association between SA-β-gal activity and the IHC detection of SA-β-gal in frozen tissues, in FFPE samples the co-localization of SA-β-gal and the senescence-associated protein $p16^{INK4a}$ was only observed in about 20% of the samples. Moreover, the expression of senescence-related proteins, $p16^{INK4a}$ and $p21^{WAF1}$, did not correlated with relapse (p=0.221 and p=0.357, respectively).

3.2 Materials and Methods

Human samples

This study was approved by the Ethics Commission of Hospital de Santa Maria – CHLN, and all patients signed an informed consent. 35 samples from patients with rectal cancer treated with neoadjuvant CT and RT were included. Tissue specimens collected at surgery were included in OCT Compound (Sakura) and snapshot frozen at -80 °C, or fixed in formalin and embedded in paraffin, by the Pathology Department of Hospital de Santa Maria, CHLN.

Cell culture

HCT116 human colon cancer cell line (ATCC) was cultured in McCoy's 5A Modified Medium (Gibco, Invitrogen) supplemented with 10 % fetal bovine serum (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 1 % non essential amino acids (Gibco), under standard conditions. $2.0x10^5$ cells were seeded in 60 mm diameter plates with cover slips and continuously exposed to 5 µM 5-FU (Accord Farmacêutica Lta.) for 7 days (medium was replaced by fresh medium with drug every 48 hours) in order to induce cellular senescence.

Immunofluorescence

48 hours after treatment with 5 µM 5-FU, the medium was removed and cells were washed and fixed with 1 % formaldehyde/2 % PFA, for 2 minutes at RT, washed in PBS, and permeabilized with ice cold methanol for 10 minutes. Cells were washed and incubated with human polyclonal β-galactosidase antibody (1:50; H80, Santa Cruz Biotechnology), washed in PBST (0.05 % Triton x-100 in PBS) 3 x 10 minutes, and followed by incubation with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:150; Molecular Probes). Antibodies were diluted in 0.1 % fish skin gelatin, 0.1 % sodium azide in PBS and incubated for 30 minutes at 37 °C. Cells were fixed in 4 % formaldehyde, washed and mounted in Vecta-Shield with DAPI (Vector Laboratories). Cells were visualized in a Zeiss

Axiovert 200M fluorescence microscope. Negative controls were obtained by omitting the primary antibody.

Detection of SA-β-galactosidase activity

Detection of SA-β-galactosidase activity was performed with the "Senescence Cells Histochemical Staining Kit" (Sigma-Aldrich), according to manufacturer's instructions and visualized in a Leica DM2500 bright field microscope (Leica Microsystems).

Western Blot

For Western blot analysis, total protein extracts were prepared by lysing cells in 200 µl 2 x SDS loading buffer, and heating at 95 °C for 10 minutes. Samples were loaded onto a 10 % polyacrylamide gel and transferred to nitrocellulose membranes (Whatman). Membranes were blocked for 1 hour in 2.5 % skim milk in PBST, incubated for 2 hours with the primary antibody and for 1 hour with the secondary antibody. Signal was visualized by chemiluminescence using the SuperSignal West Pico Chemiluminescent HRP Substrate (Pierce) according to the manufacturer's instructions. The following primary antibodies were used: β-galactosidase (1:200; H80, Santa Cruz Biotechnology) and β-actin (1:25,000; AC-15; Abcam) that was used as loading control. Anti-mouse IgG and anti-rabbit IgG secondary antibodies conjugated to peroxidase were purchased from Santa Cruz Biotechnology.

Immunohistochemistry

3 µm FFPE sections were deparaffinized and rehydrated. Antigen retrieval was performed in microwave oven, by boiling slides in 0.01 M sodium citrate buffer pH 6.0 (Sigma-Aldrich) for 10 minutes, 20 minutes, or in Tris-EDTA pH 9.0 for 30 minutes, for $p21^{WAF1}$ (EA10, Millipore), β-gal or Ki-67 (MIB1, Dako) immunodetection, respectively. For $p21^{WAF1}$ immunostaining, slides were kept in antigen retrieval solution allowing cooling for 20 minutes and cells were permeabilized with 0.05% Triton x-100 for 10 minutes with gentle agitation. 5 µm cryostat sections were fixed in ice cold acetone for 2 minutes, and washed 2 x 2 minutes in wash buffer (0.005 % Triton x-100 in PBS). Endogenous peroxidase was blocked by incubation in 1.5 % or 0.3 % hydrogen peroxide in methanol (FFPE or frozen

sections, respectively) for 10 minutes. Total protein was blocked with Protein Block reagent (Dako) for 15 minutes. Primary antibodies were diluted in Antibody dilution solution (Dako) and incubated for: anti- $p21^{WAF1}$, 1 hour at RT (1:50) followed by two washes of 5 minutes in wash buffer, for anti-β-gal, 1 hour at RT (1:50) followed by 15 minutes in wash buffer with gentle agitation for FFPE sections, or 1 hour at 4 °C (1:100) followed by 5 minutes in wash buffer for frozen sections; and anti-Ki-67, 30 minutes at RT (1:100) followed by 2 x 3 minutes in wash buffer for FFPE sections. Detection was performed with EnVision™ Dual Link HRP Rabbit/Mouse (Dako) for 30 minutes at RT. Slides were rinsed in wash buffer and incubated with 3,3'-diaminobenzidine (Dako) for 5 minutes $p16^{INK4a}$ immunodetection (CINtec® Histology Kit) was performed according to the manufacturer's instructions. All sections were counterstained with hematoxylin and visualized in a Leica DM2500 bright field microscope (Leica Microsystems).

Tissue analysis was performed by an independent Pathologist. The German Immunohistochemical Scoring System was applied for $p16^{INK4a}$. Tissues were evaluated for the intensity of the nucleic and cytoplasmic staining (0- no staining, 1-weak staining, 2-moderate staining and 3- strong staining) and the proportion of stained cells (0 < 1 %, 1-1– 25 %, 2- 26–50%, 3- 51–75% and 4- 76– 100%). The final score was determined by multiplying the intensity score with the proportion score, giving a minimum score of 0 and a maximum score of 12. For $p21^{WAF1}$ the obtained score represents the percentage of positive cells: 0 < 1%, 1- 1-5% and 2- > 5%.

Statistical Analysis

Logistic regression analysis was performed in IBM SPSS Statistics software. The Student's *t* test, the Chi square test and the Fisher's exact test were used throughout as appropriate. The level of statistical significance was set at $p < 0.05$.

3.3 Results and Discussion

Senescence-related studies are limited to the use of fresh frozen material and the cytochemical detection of SA-β-gal activity at pH 6.0. To retrospectively study archived FFPE samples of human rectal cancer, we first addressed the possibility to use the detection of β-galactosidase by IHC to identify senescent cells in this cohort.

3.3.1 β-galactosidase is increased in 5-FU-induced HCT 116 senescent cells

We first established an *in vitro* model of senescence using HCT 116 colorectal cancer cells. In accordance to what we previously described **(Chapter 2)** after a continuous exposure to 5.0 µm 5-FU for 7 days, SA-β-gal-positive blue cells with the typical enlarged senescence-like morphology were observed **(Figure 3.1A)**. With this model we obtained approximately 80% senescence-enriched cell cultures.

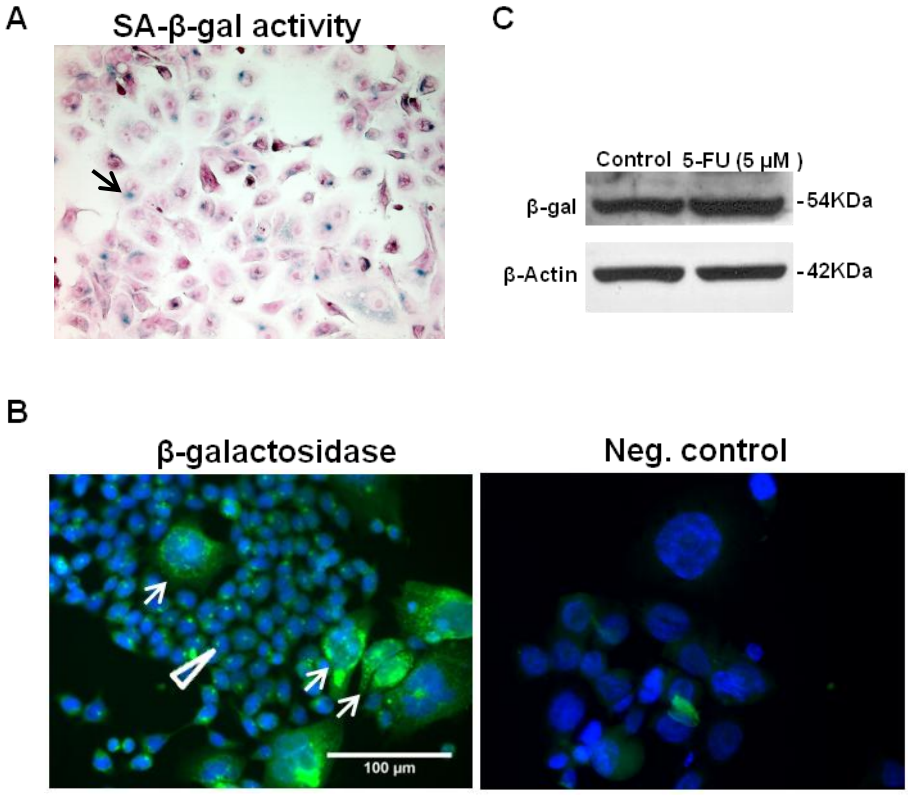


Figure 3.1- β-galactosidase expression is increased in senescent HCT116 cells.

Histochemical staining for the enzymatic activity of SA-β-Gal in HCT116 cells treated with 5 µM of 5-FU **A-** Black arrow indicates blue staining resulting from SA-β-gal enzymatic activity in typical enlarged senescence-like cells. β-gal (green) was detected by immunofluorescence **B**- Arrows point to perinuclear β-gal overexpression in enlarged and flattened senescent cells. Open arrowheads indicate lack of perinuclear β-gal overexpression in non-senescent cells. Negative control was obtained by omitting the anti-β-gal antibody. Nuclei were stained with DAPI (blue). β-gal expression was analysed by Western Blot **C**- β-actin was used as loading control.

It was previously described that the increase in SA-β-gal activity at pH 6.0 also reflects an increase in the lysosomal content of this protein, due to the gain of cellular volume, typical of the senescent phenotype [137, 178]. Therefore we hypothesized that β-gal overexpression could be used to identify senescent cells namely when the detection of enzymatic activity is not possible. To address if we could detect β-gal overexpression in senescent cells, we started by visualizing β-gal overexpression by immunofluorescence (IF). In fact we observed strong perinuclear staining in all the enlarged and flattened cells **(Figure 3.1B)**.The increase in β-gal protein was confirmed by Western blot **(Figure 3.1C)** and was consistent with previously reported results [137].

Based on these results we further analyzed whether we could detect β-gal overexpression by IHC in FFPE tissues, and especially if it could be used to identify senescent cells, in the absence of distinctive morphologic features.

3.3.2 β-galactosidase immunostaining associates with SA-β-gal activity in frozen rectal tumors

Using fresh frozen samples of human rectal cancers from patients treated with neoadjuvant CRT, we could detect SA-β-gal activity in the tumor glands **(Figure 3.2A)**. We also observed cytoplasmatic β-gal staining by IHC in the same areas of sequential sides **(Figure 3.2B)**.

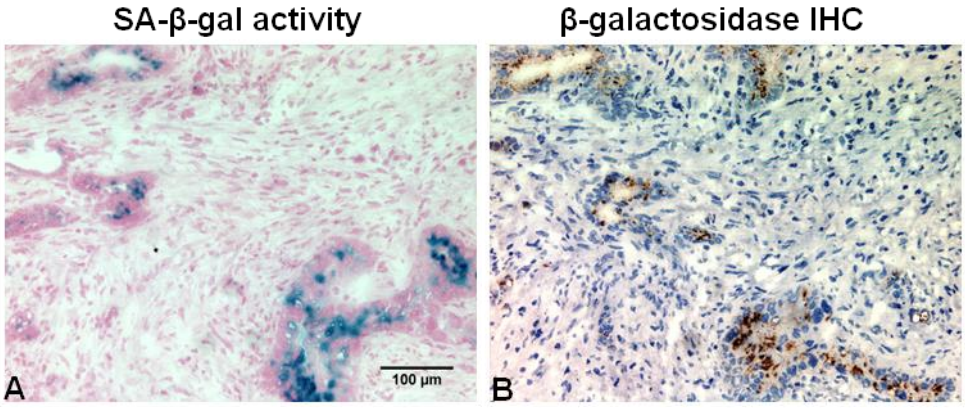


Figure 3.2- SA-β-gal activity co-localizes with β-galactosidase expression in frozen rectal cancer samples.

Sequential tissue sections from a rectal cancer specimen were used for detection of **A-** enzymatic activity of SA-β-gal at pH6.0 and **B**- β-gal by IHC.

Based on this results, we hypothesized that detection of β-gal by IHC could be a potential marker of cellular senescence. Therefore, we proceed by optimizing a protocol for IHC detection of β-gal in FFPE tissues of rectal cancer.

3.3.3 Positive β-gal/p16INK4a cells are heterogeneously found among rectal cancer tissue specimens

We observed specific cytoplasmatic β-gal staining in tumor cells from FFPE tissues of rectal cancer (n=35) **(Figure 3.3A)**.

To further characterize the specificity of the immune signal, next we assessed the co-localization of β-gal with $p16^{INK4a}$, a Cdk inhibitor previously reported as a marker of senescence [143].

By analyzing sequential slides we observed a co-localization between β-gal and $p16^{INK4a}$ **(Figure 3.3B)**, suggesting that β-gal and $p16^{INK4a}$ positive cells are in fact senescent cells. To reinforce this data, we further confirmed the unproliferative state of β-gal/$p16^{INK4a}$ positive cells, that were negative for Ki-67 immunostaining **(Figure 3.3C)**. Moreover, positive β-gal/$p16^{INK4a}$ and negative Ki-67 cells, were shown to be non-apoptotic cells by TUNEL assay (data not shown).

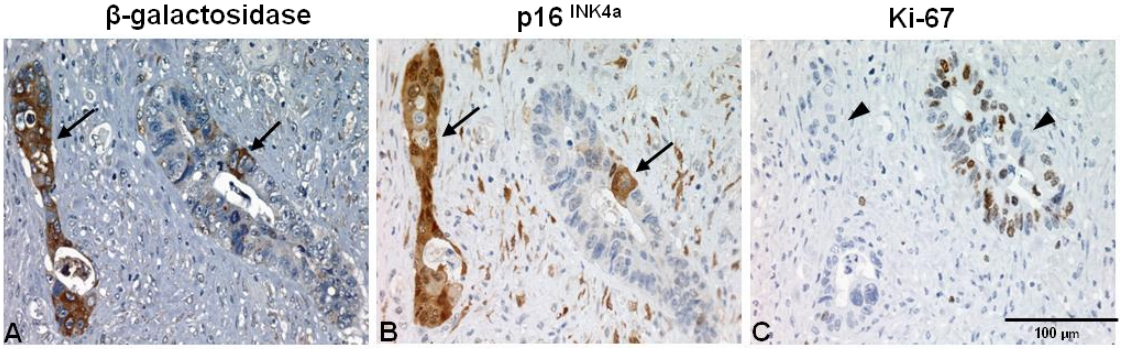


Figure 3.3- β-galactosidase co-localizes with p16INK4a in FFPE tissues.

Sequential FFPE sections of human rectal cancer were stained by IHC for **A-** β-gal; **B-** p16INK4a, and **C-** Ki-67. Arrows indicate co-localization between expression of β-galactosidase and p16INK4a. Cells negative for Ki-67 are indicated by arrowheads.

However, specific co-localization between β-gal and $p16^{INK4a}$ was only observed in 20% (7/35) of the cases. 63% of the samples showed a partial co-localization of β-gal and $p16^{INK4a}$, 10% of the samples were β-gal positive / $p16^{INK4a}$ negative, 3% were $p16^{INK4a}$ positive / β-gal negative, and 3% of the samples were negative for both markers.

In conclusion, the immunodetection of β-gal/p16^{INK4a} was shown to be inconsistent among the samples tested.

Moreover, it is described that from the four members of INK4 family, p16^{INK4a} is inactivated in a large proportion (>30%) of all human tumors [180], leading to false interpretations of the results in cases highly or poorly stained for p16^{INK4a}, respectively. This can account for the lack of co-localization with β-gal positive cells in some cases.

We also observed that variable fixation periods during sample processing, can affect the immunodetection of β-gal, suggesting that β-gal IHC staining must be optimized according to the sample processing protocol. Fixation time was not uniform across our cohort, which may have contributed to observation of p16^{INK4a} positive / β-gal negative cells.

The potential biases in β-gal IHC staining lead us to decide that it is not, so far, a suitable marker for senescence in FFPE studies.

In the absence of an unambiguous single marker, it is recommend the use of at least two senescence-related proteins for senescence identification [53]. Thus, to further analyze the potential correlation between senescence and relapse in human rectal cancers we decided to use two independent markers, p16^{INK4a} and p21^{WAF1}. Although p16^{INK4a} is not a universal marker of senescence it was previously shown to correlate with senescence both *in vitro* and *in vivo.* p21^{WAF1}, other Cdk inhibitor, also proved to be a viable marker in specific contexts like senescent hepatocytes from non-alcohol-related fatty liver disease [135, 144].

3.3.4 There is no correlation between senescence markers p16^{INK4a} or p21^{WAF1} and cancer relapse

To address if cellular senescence could be related with poor clinical outcome, we performed a retrospective analysis in a cohort of patients diagnosed with rectal cancer, positive regional lymph nodes (pN1-2) and treated with neoadjuvant CRT (n=35). Patients were stratified in two groups according to disease relapse or not at least three years after the diagnosis (n=23 and n=12, respectively) **(Table 3.1)**. There were no differences regarding major clinicopathologic parameters that could influence the risk of relapse.

Table 3.1- Patient and tumor characteristics and treatment factors (n=35)

Characteristics		With relapse	Without relapse	*p* value
	No. of patients (%)	23 (66)	12 (34)	
Age				
	Median	61	66	0.3131*
	Range	33-77	55-83	
Sex (%)				
	Male	14 (61)	8 (67)	1.0000£
pT classification (%)				
	pT1	-----	2 (17)	0.4210#
	pT2	7 (30)	3 (25)	
	pT3	13 (57)	6 (50)	
	pT4	3 (13)	1 (8)	
Clinical stage (%)				
	IIIA	4 (17)	5 (42)	0.7768#
	IIIB	14 (61)	6 (50)	
	IIIC	5 (22)	1 (8)	
K-*ras* status (%)				
	Mutated	6 (26)	5 (42)	0.4506£
	Wild type	17 (74)	7 (58)	
Adjuvant chemotherapy (%)				
	Fluoropyrimidine	5 (22)	4 (33,3)	0.5572#
	Oxaliplatin-based	12 (52)	4 (33,3)	
	Other	6 (26)	4 (33,3)	
Time to progression (months)				
	Median	20	-----	
	Range	3-35	-----	
Follow-up (months)				
	Median	-----	58	
	Range	-----	35-93	
Positive IHC (%)				
	$p16^{INK4a}$	18 (78)	12 (100)	0.5673£
	$p21^{WAF1}$	17 (74)	7 (64)	

*Student's *t* test; £Fisher's exact test; #Chi square test

Next we analyzed by IHC the expression of $p16^{INK4a}$ and $p21^{WAF1}$. The expression of $p21^{WAF1}$ was detected in a reduced number of cells *per* tumor sample and was not observed in all samples, while $p16^{INK4a}$ was broadly stained in 77 % of the analyzed samples. Data from logistic regression analysis did not showed a significant correlation between $p16^{INK4a}$ (*p*=0.221) or $p21^{WAF1}$ (*p*=0.357) and relapse **(Table 3.2)**.

Table 3.2- Logistic regression analysis to correlate independently $p16^{INK4a}$ and $p21^{WAF1}$ immunohistochemical scores with relapse (n=35).

	Odds ratio	95% CI		*p* value
$p16^{INK4a}$	0.885	0.727	1.077	0.222
$p21^{WAF1}$	0.552	0.156	1.953	0.357

We believe that two important factors may have impaired an accurate analysis, justifying further research. First, in this retrospective study the number of enrolled patients was small (n=35). This study only enrolled patients diagnosed prior to 2009 (with at least three years of follow-up), and about 20 new patients with rectal cancer are admitted annually at the Oncology Department of HSM, for who the standard of care treatment includes neoadjuvant CRT. Secondly, as above described, the lack of a universal marker or protocol to identify senescent cells in FFPE samples persists. Finally, we cannot exclude that the paradoxical effects of SAS compounds, as observed in our previous experiences, could be responsible for the lack of correlation between the TIS markers and the risk of relapse.

The possibility to identify senescent cells in fixed tissues could represent an important tool to investigate the role of senescence not only in neoplastic diseases but also in chronic inflammation and age-related pathologies. Since the particular morphological and biochemical characteristics observed in senescent cells *in vitro* are not distinguishable in rectal cancer archived samples, our efforts have been towards the validation of a method applicable to FFPE tissues. Our results suggest that the detection of β-gal protein overexpression by IHC could be a useful method to identify senescent cells in FFPE tissues, although requiring sample-specific optimization of the IHC protocol.

In summary, the search for a new universal marker of senescence is still a major goal as it is the clear determination of senescence's role in CRC patient´s outcome. For this purpose the cohort of patients is being enlarged in order to obtain a more representative group of samples with higher statistical power.

Given the clinical benefit that can be achieved with neoadjuvant chemotherapy, it will be important to determine the effects of senescence on tumor progression, which may lead to modulate or even to create new therapeutic strategies.

CHAPTER 4

GENERAL DISCUSSION

4.1 General discussion

The work presented in this thesis was performed in the Clinical and Translational Oncology Research Unit (Luis Costa Lab), in strict collaboration with the Oncology Division from Hospital de Santa Maria-CHLN.

Translational science is based on real medical questions and needs ("from the bed-side to the bench") and the ultimate goal is to apply the acquired knowledge in the finding of new perspectives to deal with a disease that affects not only the patients' group study, but thousands of people worldwide ("from the bench to the bed-side").

The main goal of this work was to determine the effects of TIS in rectal cancer.

Globally, the results obtained during this research project have provided a deeper knowledge about the possible non-cytotoxic parallel effects of chemotherapy in rectal cancer patients. Data is presented and discussed in **Chapters 2 and 3.** In this chapter the full implications of our work will be described, to a better understanding of tumor biology and in particular of tumor response to chemotherapy.

Tumor relapse, local or distant, occurs due to treatment resistance or under prognosis, and is still the leading cause of death of cancer patients. Therefore, development of effective and personalized therapy is one of the main challenges in medicine in order to improve disease free survival of cancer patients.

In advanced rectal cancer, relapse rates remains as high as 15 to 20%, despite the efforts to improve local control and distant recurrence, which suggests that residual malignant cells either in the site of primary tumor or already in circulation may require more effective and additional therapeutics.

Pathological staging following neoadjuvant CRT and surgery is the most important prognostic factor to predict relapse in rectal cancer, and is based on tumor extension through the rectal wall and spread to the regional lymph nodes [20]. More recently, the use of TRG has demonstrated the relevance of the presence of viable tumor cells after CRT as a prognostic factor [22]. However, it is still not possible to identify the patients that will have resistance to CT, and complete or partial tumor response to neoadjuvant

treatment [181]. Therefore, the identification of novel predictive biomarkers of response to treatment and predictive of relapse is extremely important in order to improve individualized therapeutic strategies and increase cure rates.

Currently, cellular senescence is considered an important tumor suppressor mechanism by limiting uncontrolled proliferation of cells and preventing neoplastic transformation [116]. However, it was previously shown that senescent cells acquire a SASP, a secretome largely composed by inflammatory cytokines/chemokines, growth factors and extracellular-matrix-remodeling proteins, secreted to the external environment that may promote tumor progression [129]. Throughout the course of the present work, we established an *in vitro* model of TIS in CRC and also analyzed human samples from rectal cancer, to address the effect of senescent tumor cells, rather than associated stromal cells, in tumor behavior.

Presently, 5-FU is still widely used to treat different cancers, including breast and CRC, and is the main CT drug recommended in the neoadjuvant setting for treatment of advanced rectal adenocarcinoma [3, 11].

Pharmacokinetic profile of the continuous Iv infusion of 5-FU is well established [39-41, 182], and Cpss values range from 0.39 µM to 66.3 µM, probably due to different metabolic rates [40, 41]. In this work we established an *in vitro* model of TIS by mimicking the effects of clinically relevant concentrations of 5-FU. In this context, HCT 116 colon cancer cells were continuously exposed to a low concentration of 5-FU, specifically 5.0 µM, for seven days **(Figure 2.1)**. Our goal was to determine the effect of such low concentrations in cellular outcome, since standard CT regimens, besides cell death, can induce TIS [42, 43, 54].

While senescence's importance is highly recognized, there are still many key questions to be answered. Could the imbalance of several pro-inflammatory cytokines, epithelial growth factors and tissue remodeling enzymes secreted by senescent tumor cells interfere with tumor cell capacity to become undifferentiated, survive and proliferate? Or could instead sensitize tumor cells to chemotherapy, improving drug response? Could senescence be related with cancer relapse?

We hypothesized that the pro-tumorigenic effect of senescence could be related with the high relapse rates in rectal cancer.

Using a cytokine array, we first analyzed the cytokine profile of the CMs obtained either from HCT 116 senescent or non-senescent cells (control). The results showed the exclusive presence of several cytokines like IL-8, MIF, VEGF, and uPAR in the CM from senescent cells. Moreover, four cytokines (e.g. EMMPRIN, cystatin C, LCN2 and TGF-α) were described as part of the SASP for the first time **(Figure 2.2A** and **Figure 2.2B)**.

Afterwards we quantified IL8 from both collected CMs and the results showed a significant increase of IL8 in CM from HCT 116 senescent cells in accordance with cytokine array results. Therefore we named it SAS-medium **(Figure 2.2C)**, and used it in subsequent analysis.

The functional effects of SAS-mediums from different sources have been previously studied, becoming evident that senescent fibroblasts acquired a powerful secretome capable of promoting tumorigenesis [118, 128-131, 172]. Recently, some reports shed some light about the effects of different SAS in distinct neoplastic contexts [133, 183, 184].

Considering that the overexpression of the cytokines found in SAS-medium has been implicated in CRC, contributing to cancer progression [185-187], we subsequently investigated the effects of the SAS-medium from TIS tumor cells in non-senescent tumor cells of three different CRC cell lines (two colon cancer cell lines- HCT 116 and SW48, and one cell line from rectal cancer- SW837).

We provide significant *in vitro* evidences that the SAS-medium stimulated cells to proliferate **(Figure 2.3)**, induced a loss of E-cadherin, a specific epithelial cell marker, significantly increased the expression of EMT-associated genes like *Snail*, *Slug, ZEB1A*, *vimentin* and *fibronectin* **(Figure 2.4A** and **Figure 2.4B)**, and significantly increased cell invasion **(Figure 2.4C)**.

During EMT, cells undergo through the process of dedifferentiation, losing the epithelial markers and apical-basal cell polarity leading to shape alterations. The gain of

mesenchyme-like phenotype comprises actin cytoskeleton reorganization and cytokeratin intermediate filaments are replaced by vimentin intermediate filaments. Thus, cells change from a cuboidal to a spindle shape, promoting cell migration and invasion. Therefore, the dedifferentiation of cells during EMT is considered a key process, in tumor progression, facilitating tumor cell dissemination and metastasis [177, 188].

Therefore, our data suggests that SAS components from epithelial CRC cells contributes to an acquisition of mesenchymal features and, by this mean, to a more aggressive and invasive phenotype.

Next, we tested SAS-medium's effects in cellular response to 5-FU. We showed for the first time that SAS increased chemosensitivity to 5-FU by reducing 5-FU IC50 values in all tested cell lines **(Figure 2.5A)**. An ongoing prospective study which details will be provided further ahead in this chapter, will allow us to clarify whether the reduced IC50 values are clinically relevant or not.

It was previously shown that in CRC, expression of TS enzyme was correlated with the response to 5-FU-based therapies with improved responses in patients with low TS expression [33]. Therefore, we addressed the TS expression and interestingly we found a significant decrease in TS expression in cells incubated with SAS-medium **(Figure 2.5B)**. Additional data from cell cycle analysis showed an increased number of cells accumulated in S-phase when incubated with SAS-medium **(Figure 2.5C)**.

These results lead us to hypothesize that SAS-medium also sensitizes tumor cells to chemotherapy, improving drug response.

Furthermore, we obtained similar results using CM from doxorubicin-induced senescent HCT 116 cells, leading us to conclude that the observed effects were independent of the drug used to induce senescence **(Supplementary Figure S2.1, Supplementary Figure S2.2** and **Supplementary Figure S2.3)**.

Subsequently we questioned about the physiopathological relevance of the above described results. To address this we used frozen rectal cancer specimens, from patients treated or not with neoadjuvant CRT, collected during standard of care surgery. Next we

randomly selected and isolated clusters of tumor cells free of stromal components, by laser capture microdissection. We analyzed the isolated tumor cells for specific mRNAs and importantly we verified that the expression of the senescence-related gene *p21$^{Waf1/Cip1}$*, of the SAS component *IL8*, and EMT-related genes like *Snail*, *Slug* and *vimentin* was increased in samples from patients that were submitted to treatment before surgery **(Figure 2.6A)**.

Moreover, we specifically isolated and compared the expression of these genes in clusters of tumor cells enriched in senescent cells with their expression in tumor cells located far from the senescent cells. Our results provide clear evidence about the local effects of SAS from senescent tumor cells, since the expression of *IL-8* and EMT markers decreased in the tumor cells that distance 700 µm from senescent cells **(Figure 2.6B)**.

Therefore we hypothesize a dual effect of SAS from senescent tumor cells. If on one hand we showed that the secretome of senescent tumor cells promote more aggressive phenotypes by inducing dedifferentiation of epithelial transformed and proximal tumor cells with potentially implications in cancer relapse, on the other hand, we showed evidences of an anti-tumor effect by sensitizing cells to 5-FU treatment and therefore improving drug response.

Paradoxical effects of SAS compounds have been described in the past. If on one hand the SAS favors malignancy in neighboring cells, on the other hand, it may reinforce growth arrest and protect against cancer by activating the immune response, inducing the clearance of damaged and pre-neoplastic cells [125, 126, 128].

In addition, some authors support the theory that senescence may have evolved as an example of antagonistic pleiotropy, since it provides beneficial traits during the reproductive age of an individual (tumor suppression via growth arrest), but causes deleterious effects later on in life (aging and cancer via SAS) [116].

However, this is the first study providing evidence that senescent cancer cells influence the tumor microenvironment by promoting EMT via short range interactions in human rectal cancer samples. Moreover, we report the first evidences of increased

chemosensitivity in cells from a CRC cell line when incubated with SAS in combination with 5-FU.

Distinct quantitative and qualitative differences in SAS were already described [118]. As expected, the effects of senescent cells within the tumor microenvironment are complex and highly dependent on physiological context demonstrating how complex and heterogeneous the senescent biological program can be. Therefore, we have no doubt that important information could be obtained with the complete screening of SAS-medium composition. This information would be critical to identify potential new therapeutic targets and to develop new strategies aiming the inhibition of the cancer-promoting components of the SAS.

Based on the obtained results, we could not determine whether cellular senescence may indeed contribute to patient outcome after anticancer therapy. The phenomenon of drug-inducible senescence in tumors treated with neoadjuvant chemotherapy requires further research in order to determine its significance in patient's outcome. We hypothesized that senescence may specifically correlate with rectal cancer relapse. Therefore we performed a retrospective study enrolling 35 patients diagnosed with advanced rectal cancer, treated with neoadjuvant CRT, and with clinical follow-up of at least three years after the diagnosis in order to investigate a possible correlation between senescence and clinical outcome of rectal cancer patients. Patients were stratified in two groups according to stable disease or relapse **(Table 3.1)**.

We then evaluated the presence of senescent cells in tumor specimens. To address the detection of senescent cells in FFPE tissues we first attempt to validate β-galactosidase detection by IHC as a new method for the identification of senescent cells applicable to FFPE tissues (**Chapter 3).**

However, despite the promising preliminary results obtained in frozen tissues, where we showed a clear association between SA-β-gal activity and the immunohistochemical detection of β-gal, in FFPE tissues the results were inconsistent **(Figure 3.2** and **Figure 3.3)**. In the absence of one 'gold standard' marker, and despite the fact that these are not universal markers of senescence [180], we selected the expression of Cdks inhibitors

$p16^{INK4a}$ and $p21^{Waf1/Cip1}$, whose increased expression mediates cell cycle arrest in senescence. Moreover, these two proteins were already used to identify senescent cells in different contexts [135, 138, 144, 159].

The expression of these two senescence-associated markers was not uniform across tumor samples. In fact, $p21^{WAF1}$ was detected in a small proportion of tumor cells *versus* the entire tumor cell population, while $p16^{INK4a}$ was broadly detected in the tissue sections of majority of samples. We could not find a significant correlation between the scores found by IHC for $p16^{INK4a}$ or $p21^{WAF1}$ and cancer relapse.

These results are, therefore, inconclusive. Either due to the small number of patients enrolled for this study, or to the lack of a reliable marker of senescence, we believe this study should be enlarged and repeated with other markers of senescence.

Over the past few years cellular senescence has been clearly identified in a variety of *in vitro* cancer models. Consistently, evidences show that senescence response can be invoked by a wide spectrum of agents in specific conditions, and in fact contribute to treatment outcome [54, 156]. However, due to heterogenic findings, the presence of senescence often failed to accurately predict the clinical outcome of cancer patients.

Breast cancer cells in a mouse model of doxorubicin-induced senescence showed to be highly resistant to apoptotic stimuli and to likely contribute to cancer relapse through its autocrine/paracrine activity [157]. Furthermore, senescence in human samples from patients treated with neoadjuvant CT is correlated with low OS and shorter PFS (p= 0.0098 and p= 0.022, respectively) in patients with malignant pleural mesothelioma and with a decrease in OS (p= 0.04) in cases of non-small cell lung carcinoma. On the contrary CRC patients with senescent cells detected previous treatment tend to increase TIS susceptibility and, according to the authors, have better chemotherapy responses [160].

Conventional anti-cancer CT may fail due to inaccessibility of tumors, cellular heterogeneity, or even resistance to treatment. While increasing drug concentrations could overcome the low chemosensitivity, its clinical applicability is limited due to concomitant toxicity [189].

An alternative strategy to improve the response to cancer therapeutics is the induction of cytostasis, which permanently arrests the proliferative capacity of cells without inducing cell death *per se*. In this context, the induction of tumoral senescence could represent a different approach in treatment of neoplastic diseases.

TIS could provide an effective and persistent inhibition of the tumor growth in both early and late-stage cancers rather than causing tumor regression. In addition, induction of senescence implies the use of lower drug concentrations, limiting toxic side effects. In theory, senescence induction could provide equivalent or prolonged survival with less severe side effects related to toxicity and may provide a more realistic goal for the chronic management of some cancers [42, 190].

Again, there are controversial opinions since senescent cells remain viable and metabolically active, acquiring a new transcriptional profile – SASP. We demonstrated that the SAS may inhibit the growth of the surrounding cells or, alternatively, may be implicated in growth and invasion promoting effects, increasing the risk of cancer relapse. For those reasons, until now there are no evidences of pro-senescence cancer therapies that exploit SAS components at either experimental or preclinical stage [191].

While therapies that induce cellular senescence can be a potential strategy to fight cancer, the accumulation of senescent cells on aging tissues may fuel the development of late life cancer. Studies in aging have suggested an association between age-related senescence and the promotion of chronic inflammation and carcinogenesis in surrounding tissues [192]. In this context, some authors are pointing out the beneficial aspects of quantitative elimination of senescent cells as therapeutic priority, either through the immune system or through biological or chemical approaches. A recently published report unveiled the hypercatabolic nature of TIS, an approach therapeutically exploitable by synthetic lethal metabolic targeting where it was shown that blocking metabolic demands of TIS induction, like glucose, led to selective elimination of senescent cells, prompted tumor regression and improved treatment outcomes [193].

The induction of senescence as a new approach in cancer therapy requires intensive research and the clear identification of the pathways that regulate senescence and of the specific senescence-inducing agents.

To conclude, the results obtained in this work suggest that neoadjuvant therapy may not only exert its effectiveness by downstaging or reducing tumor cell proliferation, but also by qualitatively altering the phenotype of cancer cells and ultimately increasing drug perception of the remaining apoptosis resistant cells. Given the additional clinical benefit that can be achieved by neoadjuvant CT in rectal cancer, long-term prospective data on the prognostic impact of TIS is still missing.

For that reason, during the course of this project, we started to prospectively collect serum samples from patients treated with intravenous infusion of 5-FU. So far we have a total of 75 serum samples corresponding to 25 patients with rectal cancer treated with neoadjuvant CRT. Blood samples were collected at baseline, i.e., before treatment initiation, after 48 hours of continuous drug administration and in the last day of treatment. It is our main goal to determine the serum concentrations of 5-FU in the different time points and correlate it with tumor cells fate on the surgical specimen of the same patient. These results will allow the stratification of patients based in the ability to produce tumor cell senescence following chemotherapy in neoadjuvant settings and improve patient-oriented therapy outcome.

Final remarks

The findings compiled in this thesis can impact the current scenario of rectal cancer treatment strategies.

TIS may be a predictive factor for treatment outcome and the either pre-existing or CT-induced senescence should be addressed in order to improve and personalize cancer treatments.

This work focused in rectal cancer and it only became possible not only due to the truly commitment of the patients by giving their consent to study their biological samples

(blood, biopsies and surgical specimens), but also by the collaboration between basic scientists, oncologists, surgeons, pathologists and even nurses and technicians. Through our work and through the communication bridge that we established everyday between basic science researchers and health care professionals, we expected to give our contribution for the translational cancer research field.

We have summarized the evidences indicating a dual role of SAS from TIS tumor cells in non-senescent tumor cells both in CRC cell lines and in samples from CT-treated patients with advanced rectal cancer. **We propose that** despite SAS form senescent cancer cells influence the tumor microenvironment by promoting EMT in a specific range of action, favoring tumor progression, it also sensitizes tumor cells to chemotherapy, improving drug response. We also suggest further *in vivo* studies, not only increasing the number of samples in the clinicopathologic study, but also additional prospective data that will shed light if drug-inducible senescence might even act as the essential prognostic factor in determining tumor control *versus* relapse. **Future studies, aimed at** providing a greater understanding of SAS-medium composition of epithelial CRC cells to further dissect underlying signaling cascades with particular focus for the secreted factors involved in the chemosensitivity effect are required.

Some barriers still remain, including the identification of more reliable markers of senescence and continued investigations of the biological implications of senescence in the clinical outcome of patients.

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